

1984

The effect of nucleosides on the expression of the glycoprotein hormone alpha subunit and placental alkaline phosphatase in HeLa cells

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**THE EFFECT OF NUCLEOSIDES ON THE EXPRESSION OF THE
GLYCOPROTEIN HORMONE ALPHA SUBUNIT AND PLACENTAL ALKALINE
PHOSPHATASE IN HELA CELLS**

Iowa State University

Ph.D. 1984

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**The effect of nucleosides on the expression
of the glycoprotein hormone alpha subunit and placental
alkaline phosphatase in HeLa cells**

by

Richard Neil Tamura

**A Dissertation Submitted to the
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ABBREVIATIONS

8 AzaG	8-azaguanine
AraC	Cytosine arabinoside
BrdUrd	5-bromodeoxyuridine
BSA	Bovine serum albumin
Btr	sodium butyrate
cAMP	5',3'-cyclic adenosine monophosphate
Con	control
Con A	Concanavalin A Sepharose
dATP	deoxyadenosine triphosphate
dCyd	deoxycytidine
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dNTP	deoxynucleoside triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
EMS	ethyl methanesulfonate
FdUrd	5-fluorodeoxyuridine
FSH	follicle stimulating hormone
GPDH	glycerol phosphate dehydrogenase
GS	glutamine synthetase
hnRNP	heterogeneous nuclear ribonucleoprotein
hCG	human chorionic gonadotropin
HU	hydroxyurea

IBMX	3-isobutyl-1-methylxanthine
IdUrd	5-iododeoxyuridine
LDH	lactate dehydrogenase
LE	leutenizing hormone
mRNA	messenger RNA
MEM	minimum essential medium
MEMC	minimum essential medium, complete
PAP	plecental alkaline phosphatase
PEG	polyethylene glycol
FBS	phosphate buffered saline
PFO	2,5 diphenyloxazole
POPOP	1,4-bis-2-(5-phenyloxazolyl)-benzene
RIA	radioimmunoassay
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
Thd	thymidine
TCA	trichloroacetic acid

INTRODUCTION

Ectopic proteins

Oncogenesis is characterized by cellular changes such as unregulated cell growth, alterations in the plasma membrane composition, cytoskeletal rearrangements, and changes in the activity of genes (Becker, 1973; Cameron and Pool, 1981; Ruddle, 1981; Sherbert, 1982). A change frequently accompanying neoplasia is the production of ectopic proteins. Ectopic proteins are gene products which are not characteristic of the cell type from which the tumor was derived. Ectopic proteins are produced both by tumors in vivo and by continuous cell lines derived from such tumors (Bower and Gordan, 1965; Lipsett, 1965; Liddle et al., 1969). Proteins which have been reported as ectopic tumor products include vasopressin (Klein et al., 1969), growth hormone (Greenberg et al., 1972), human chorionic gonadotropin (Rabson et al., 1973) calcitonin (Lunsden et al., 1980), parathyroid hormone (Greenberg et al., 1973), human placental lactogen (Rabson et al., 1973), alpha-fetoprotein (Abelev et al., 1967), carcinoembryonic antigen (Gold and Freedman, 1965), placental alkaline phosphatase (Stolbach et al., 1969), ACTH (Liddle et al., 1962), and isoferritins (Drysdale and Singer, 1974). Although tumor-dependent synthesis of these proteins has not always been rigorously demonstrated, the evidence for this phenomenon is strong which suggests that it may be a general feature of neoplasia (Odell et al., 1977; Bower and Gordan, 1965).

While ectopic protein synthesis may be common to neoplasia, it is not a random event, and particular ectopic proteins tend to be associated with specific tumors (Azzopardi and Williams, 1968; Rosen et al., 1980). For example, ACTH production is often associated with oat cell carcinoma of the bronchus and parathyroid hormone (PTH) with squamous cell carcinoma. Also, certain ectopic hormones tend to be produced together (e.g., oxytocin and arginine vasopressin), suggesting that their expression may be co-ordinately regulated.

Ectopic proteins are very similar to their normal counterparts in structure and function. However, differences in isoelectric points, electrophoretic mobility, and carbohydrate content indicate that they are not identical (Weintraub et al., 1975; Orth et al., 1973; Neuwald and Brooks, 1981). Many ectopic hormones are biologically active and result in clinical syndromes characteristic of aberrant hormone production such as Cushing's syndrome (ectopic ACTH), hypercalcemia (parathyroid hormone), and gynecomastia (gonadotropin). Enhanced hormone production may also arise from tumors of endocrine glands, in which case, synthesis is eutopic. It is important to be able to distinguish between the paraneoplastic effects of ectopic hormone production and production due to endocrine gland dysfunction. In at least three cases, extirpation of the normal endocrine gland was performed because the initial diagnosis was incorrect (Scholz et al., 1973; Mattingly et al., 1964; Mason et al., 1972). The study of ectopic proteins may reveal differences with the normal product to aid diagnosis.

Goldstein et al. (1980) have observed the presence of PAP activity in the endocervix and cervical mucosa of non-pregnant women with no evidence of malignancy. This report raises the question of whether cervical tumor PAP activity (e.g. HeLa) is truly ectopic. Nevertheless, PAP activity has been observed in association with a number of different types of cancer (Fishman et al., 1968; Nathanson and Fishman, 1971; Ghosh et al., 1972; Rosen et al., 1975). Fetal intestinal alkaline phosphatase has also been found in a number of tumors (Suzuki et al., 1975; Higashino et al., 1977).

Similarly, low levels of hCG have been observed in normal tissues other than placenta (Yoshimoto et al., 1977). In contrast to the placental hormone, material from liver and colon extracts seemed to contain little or no carbohydrate as determined by chromatography on Con A coupled to agarose. In addition, hCG was observed to have a low carbohydrate content in tumors where production was low and a high carbohydrate content in tumors with high production (Yoshimoto et al., 1979). These results were interpreted to indicate that the peptide sequence of hCG could be synthesized by both normal and neoplastic tissues and that the rate of ectopic hormone production may result from concomitant high rates of glycosylation.

Despite this possible ambiguity, measurement of ectopic protein levels in the circulation of patients may be used in the diagnosis of cancer as well as to monitor the effectiveness of therapy to eradicate the tumor. That is, if the synthesis of the protein is indeed tumor-

dependent, then the circulating levels should correspond with the growth or regression of the tumor. Thus, the study of ectopic proteins is significant because of their role in the biology, pathology, and treatment of tumors. Also, the study of factors affecting the production of these proteins may elucidate important regulatory events.

Human chorionic gonadotropin

This study examines the production of human chorionic gonadotropin (hCG) and placental alkaline phosphatase (PAP) in human cervical carcinoma (HeLa) cell lines; hence, a brief description of each of these proteins will be presented.

Human chorionic gonadotropin is a glycoprotein hormone synthesized primarily by the syncytiotrophoblast cells during pregnancy. It functions in the maintenance of the corpus luteum and may also promote gonadal development in the fetus.

The glycoprotein hormones hCG, TSH, LH, and FSH comprise a family of proteins which are composed of two non-identical subunits designated α and β . The α subunit is the same for all of these hormones while the β subunit is unique and conveys the biological activity of each hormone. The structure of hCG has been studied by a number of groups (Bell et al., 1969; Bahl, 1969a; Bahl, 1969b; Merz et al., 1974). The alpha subunit of hCG (hCG- α) has a M_r of about 14,900 while the beta subunit (hCG- β) has a M_r of about 23,000 as computed from the chemical composition (Bellisario et al., 1973; Carlsen et al., 1973). Both subunits are about 30% by weight carbohydrate which is linked to either

asparagine or serine residues. The amino acid sequence (Bellisario et al., 1973; Carlsen et al., 1973; Morgan et al., 1975) and the carbohydrate structures (Bahl, 1969b; Kennedy and Chaplin, 1976; Endo et al., 1979) of both subunits have been determined.

The glycosylation and secretion of hCG and its subunits has been studied by Ruddon et al. (1979a; 1979b; 1980; 1981) in the JAR (a choriocarcinoma), ChaGo (a broncogenic carcinoma), and HeLa cell lines. The hCG- α subunit has intermediate glycosylated forms with M_r of 15,000 and 18,000 and a M_r of 22,000 for the mature form as determined by SDS-polyacrylamide gel electrophoresis. The glycosylated intermediates of hCG- β have M_r of 18,000 and 24,000 while the mature protein has a M_r of 34,000. The rate limiting step in the glycosylation of hCG in JAR cells may involve the action of α -mannosidase(s) (Ruddon et al., 1981) with terminal glycosylation and secretion occurring rapidly thereafter.

The production of hCG has been detected in the serum of patients with a variety of tumors (Neville, 1982) as well as a number of tumor cell lines (Ghosh and Cox, 1976; Kanabus et al., 1978; Kornfeld and Kornfeld, 1976; Rabson et al., 1973; Tashjian et al., 1973). Synthesis of hCG is autopic if the cell line was derived from a trophoblastic tumor, such as JAR choriocarcinoma cells, and ectopic if the cell line was derived from non-trophoblastic tumors such as ChaGo broncogenic carcinoma cells and HeLa cervical carcinoma cells.

The synthesis of hCG subunits is often unbalanced in vivo (Vaitukaitis et al., 1976; Weintraub and Rosen, 1973; Rosen and

Weintraub, 1974) as well as in vitro (Tashjian et al., 1973; Chou et al., 1977; Ruddon et al., 1979b) with hCG- α production frequently in excess of hCG- β . During pregnancy, excess hCG- α is synthesized and the amount of hCG- β becomes the limiting factor in the production of holo-hCG (Vaitukaitis et al., 1976). This unbalanced synthesis of hCG and its subunits during pregnancy can be accounted for by the steady state levels of the corresponding mRNAs (Boothby et al., 1983).

Recently, the mRNAs encoding both hCG- α and hCG- β have been isolated and utilized to synthesize and clone cDNAs for both subunits (Landefeld et al., 1976; Daniels-McQueen et al., 1978; Fiddes and Goodman, 1979; Fiddes and Goodman, 1980; Boothby et al., 1981). From human-mouse hybrid cells it has been determined that two human chromosomes are required for intact hCG production (Bordelon-Riser et al., 1979; Kohler et al., 1980). The common glycoprotein subunit appears to be encoded by a single gene (Boothby et al., 1981; Fiddes and Goodman, 1981) apparently on chromosome 18 (Hardin et al., 1983), while the hCG- β subunit may be encoded by a minimum of eight genes (Boorstein et al., 1982). The number of apparent hCG- β genes may reflect the degree of sequence homology among the various genes coding for the different glycoprotein beta subunits.

Placental alkaline phosphatase

Alkaline phosphatases are a group of enzymes which catalyze the hydrolysis of monophosphate esters at alkaline pH optima. Human alkaline phosphatase consists of isozymes characteristic of specific

tissues and developmental stages. These isozymes are membrane bound glycoproteins which can be differentiated by their electrophoretic mobility (Harris and Hopkinson, 1976; Robinson and Pierce, 1964), thermostability (Neale et al., 1965; Moss et al., 1972; Petit-Clerc, 1976), sensitivity to L-amino acids (Mulivor et al., 1978b), and by immunological methods (Boyer, 1963; Sussman et al., 1968; Komoda and Sakagishi, 1976; McKenna et al., 1979).

The various human isozymes appear to be encoded by at least three structural genes: one for the placental isozyme, one for the intestinal isozyme, and one for the bone, liver, and kidney isozymes (or tissue non-specific isozyme). Several electrophoretic variants of PAP have been shown to be alleles of an autosomal locus (Robson and Harris, 1967). The other isozymes do not exhibit such variants and are presumably determined by another locus or loci (Harris and Hopkinson, 1976). Also, the autosomal recessive disease of hypophosphatasia results in depressed activity of liver, bone, and kidney alkaline phosphatases but does not affect intestinal or placental alkaline phosphatase (Fraser, 1957; Danovitch et al., 1968; Mulivor et al., 1978c). Furthermore, tryptic peptide maps of the purified isozymes are similar for bone, liver, and kidney alkaline phosphatases yet distinguishable from intestinal and placental which are themselves distinct from each other (Badger and Sussman, 1976; McKenna et al., 1979; Seargeant and Stinson, 1978). Finally, inhibition studies with a variety of L-amino acids yields identical profiles for liver, bone, and

kidney isozymes but different profiles for placental and intestinal, which are distinct from each other (Mulivor et al., 1978a).

Alkaline phosphatase isolated from the first trimester placenta (chorionic PAP) differs from the term placental alkaline phosphatase (PAP) in its thermostability and sensitivity to L-homoarginine and L-phenylalanine (Fishman et al., 1976). A similar developmental difference exists between fetal and adult intestinal alkaline phosphatase, but not with fetal and adult liver, bone, or kidney (Mulivor et al., 1978a).

The term placental isozyme of alkaline phosphatase is expressed by several tumor cell lines (Elson and Cox, 1969; Beckman et al., 1970; Luduena and Sussman, 1976; Benham et al., 1978; Hamilton et al., 1979a). In addition, some HeLa cell lines synthesize an isozyme more closely resembling the chorionic PAP or intestinal isozymes in its thermostability (Benham et al., 1978; Benham and Harris, 1979). Various differences in the physicochemical properties have been observed between the tumor associated PAP and normal PAP (Neuwald and Brooks, 1981).

Recently, the biosynthesis and processing of PAP has been examined in choriocarcinoma cells (Ito and Chou, 1983). Apparent molecular weights of 61,500 and 64,500 were estimated for the precursor and fully glycosylated forms of the PAP monomer synthesized in vivo. The unglycosylated monomer has an apparent M_r of 58,000. Also, a preprotein with M_r of 60,000 was observed by in vitro translation of choriocarcinoma mRNA in the absence of membranes.

Induction of Ectopic Proteins by Sodium Butyrate

Butyric acid or its sodium salt has multiple effects on a variety of cells (Prasad and Sinha, 1976; Kruh, 1982). These effects include the inhibition of cell growth and DNA synthesis (Wright, 1973; Riggs et al., 1977; Hagopian et al., 1977), alterations in cell morphology (Ginsburg et al., 1973; Deutsch et al., 1976; Altenburg et al., 1976; Mori et al., 1979; Borenfreund et al., 1980), modifications of nuclear proteins (Riggs et al., 1977; Vidali et al., 1978; Boffa et al., 1981), and changes in many protein and enzyme levels (Griffen et al., 1974; Chou and Robinson, 1977b; Fishman et al., 1974; Ghosh and Cox, 1976). Most of these alterations are freely reversible within 24 hours after removal of butyrate from the culture medium.

HeLa cells are normally round or polygonal in appearance and have a doubling time of about 24 hours. Recently, it has been shown that butyrate at mM concentrations arrests HeLa cell growth primarily at the G1/S border and also in the G2 portion of the cell cycle (Fallon and Cox, 1979). Arrest of cell growth in G1 has also been observed in other cell types (Rastl and Swetly, 1978; D'Anna et al., 1980b). Inhibition of cell growth is accompanied by the appearance of filamentous protrusions 7 to 8 hours after butyrate exposure, which develop into long spikes by 12 hours (Ginsburg et al., 1973). Electron micrographs of cells grown in the presence of butyrate show corresponding changes in ultrastructural components. For example, in virally transformed rat kidney cells exposed to butyrate, the assembly of cytoplasmic

microfilaments and microtubules along with the formation of substrate adhesive plaques and intercellular gap junctions are observed (Altenburg et al., 1976). Also, the addition of butyrate to chemically transformed rat hepatoma cells results in intermediate filaments which resemble normal filaments in their organization (Borenfreund et al., 1979; Borenfreund et al., 1980).

Several changes in plasma membrane components have been associated with butyrate action. For example, the fatty acid stimulates an increase in the levels of glycosphingolipid GM_3 in HeLa cells by elevating sialyltransferase I activity (Fishman et al., 1974; Simmons et al., 1975). This increase in sialyltransferase activity precedes the formation of cellular protrusions and upon the removal of butyrate, sialyltransferase activity decreases prior to the return of normal HeLa cell morphology (Fishman and Brady, 1976). Modification of membrane gangliosides has also been observed in transformed rat kidney cells treated with butyrate (Via et al., 1980). Another ganglioside, GM_1 , is the receptor for cholera toxin (Fishman and Brady, 1976; Bennett et al., 1976; Cuatrecasas, 1973). An increase of this receptor is observed after 20 hours of exposure to butyrate in HeLa, rat C6 glial, and Friend erythroleukemia cells (Fishman and Atikkan, 1979). Butyrate has a two-fold, concentration dependent effect on β -adrenergic receptors in HeLa cells. At concentrations greater than 2 mM, β -receptor levels are induced and functionally coupled to pre-existing adenylate cyclase molecules within 10 hours. At lower concentrations of butyrate (0.5 to

1.0 mM), the receptor levels are fully induced but are not functionally coupled with adenylate cyclase. A subsequent shift to the higher butyrate concentration initiates coupling following a brief 2 hour lag (Henneberry et al., 1977). Recent evidence suggests that butyrate induces qualitative changes in the regulatory component of the β -adrenergic system (Kassis et al., 1984).

Sodium butyrate, as well as cortisol and other compounds, increases the activity of PAP in HeLa cells. This increase in activity may result from either an increase in the catalytic efficiency of the enzyme or an increase in the biosynthesis of the enzyme. Studies by Ghosh et al. (1972) indicate that the PAP activity induced by cortisol results from an increase in the catalytic efficiency due to a decrease in the phosphorylation of the enzyme. Similar interpretations have been given for the induction of PAP activity in HeLa cells by 5-bromodeoxyuridine (Bulmer et al., 1976) and 5-iododeoxyuridine (Goz and Walker, 1978). However, contrary to these reports, Hanford and Fishman (1983) observe an induction in the biosynthesis of PAP in HeLa cells by the synthetic glucocorticoid prednisolone and butyrate. The increase in the amount of PAP is sufficient to account for the enhanced enzyme activity. Furthermore, Hamilton and Sussman (1981) and Ito and Chou (1984) observe that 5-bromodeoxyuridine and butyrate respectively induce the de novo synthesis of PAP in choriocarcinoma cells. It is unclear if this disagreement is due to technical differences or actual differences in the mechanism of induction.

In other cell lines which express two isozymes of alkaline phosphatase, the placental isozyme is induced preferentially (Nitowsky et al., 1963; Tokumitsu et al., 1979; Herz, 1973; Hamilton et al., 1979b). Occasionally, however, there is an equal induction of both isozymes (Speeg et al., 1977), and in SW-620 cells (established from a human colon carcinoma), butyrate induces the tissue non-specific isozyme but not the placental isozyme (Herz and Halwer, 1983).

In addition to alkaline phosphatase, butyrate also stimulates the production of human chorionic gonadotropin and its α and β subunits in various tumor cell lines (Ghosh and Cox, 1976; Liebllich et al., 1976; Chou et al., 1977; Ghosh et al., 1977). The amount of hCG- α secreted into the medium by HeLa cells is enhanced within 12 hours after the addition of butyrate followed by a more dramatic increase after 24 hours. The production of hCG and hCG- α varies among HeLa cell lines with respect to subunit accumulation in the medium and in the degree of induction (Liebllich et al., 1977; Chou, 1978; Cox, 1981c). Thus, relative to one another, some HeLa cell lines (2.2 and S-3) are high producers (about 70 to 100 ng/mg protein-day) and others are low producers (2 and 2.1; 10-30 ng/mg protein-day). The induction of hCG- α by butyrate in HeLa cells also varies with the medium composition (Cox and McClure, 1983).

While butyrate induces the ectopic synthesis of hCG and hCG- α by non-trophoblastic tumor cells (Chago and HeLa), it does not affect and may inhibit synthesis by trophoblastic cells (BeWo, JEG, and Reid

choriocarcinoma) where the placental hormone is a eutopic product (Chou et al., 1977; Husa et al., 1978). Ectopic and eutopic synthesis of hCG also differs with respect to subunit glycosylation (Ruddon et al., 1979a; Ruddon et al., 1979b; Ruddon et al., 1980). Only the glycosylation intermediates and not the fully processed hCG accumulates intracellularly in JEG cells. However, the fully processed hCG does accumulate intracellularly in ChaGo and butyrate treated HeLa cells. Thus, trophoblastic and non-trophoblastic cells may control the processing and secretion of hCG differently. Recently, glycosylation was reported to be significant in the butyrate induction of hCG- α in HeLa cells (Cox, 1981a; McClure and Cox, 1984) and Chang human liver cells (Morrow et al., 1983).

Butyrate elicits a variety of responses in hormone mediated processes. For example, the estradiol induction of ovalbumin and transferrin transcription in chick oviducts is blocked by butyrate and other short chain fatty acids (McKnight et al., 1980). Butyrate does not affect either estradiol binding to its receptor or transport to the nucleus. In rat C6 glioma cells, hydrocortisone induces glycerol phosphate dehydrogenase (GPDH) and glutamine synthetase (GS), and norepinephrine induces lactate dehydrogenase (LDH). Sodium butyrate inhibits the hydrocortisone mediated induction of GPDH but not of GS or the norepinephrine induction of LDH (Weingarten and de Vellis, 1980; Weingarten et al., 1981). Butyrate does not affect the binding of hormone to its receptor. In addition, butyrate alone induces GS but not

GPDH. Sodium butyrate also inhibits the prolactin induction of casein synthesis and casein mRNA accumulation in rabbit mammary explants (Martel et al., 1983). In this situation, butyrate inhibits induction at the membrane level by blocking the generation of the prolactin intracellular relay from prolactin treated membranes. Samuels et al. (1980) have studied the effect of butyrate on the nuclear level of glucocorticoid and thyroid hormone receptors in GH₁ cells. Butyrate decreases the thyroid hormone nuclear receptor without affecting total protein synthesis. Butyrate did not affect either the total cellular level or the nuclear association of glucocorticoid receptors. In other studies, the carboxylic acid prevented glucocorticoid induction of tyrosine aminotransferase in HTC cells (Tichonicky et al., 1983), but enhanced induction of PAP in HeLa cells by the synthetic glucocorticoid dexamethasone, even though the cytoplasmic receptor was decreased by 33% and the nuclear receptor by 49% (Littlefield et al., 1980).

A significant consequence of butyrate action is the modification of chromatin proteins. Riggs et al. (1977) first reported that butyrate induces the hyperacetylation of histones H3 and H4 primarily, and of H2A and H2B to a lesser extent. These modifications result from butyrate's non-competitive inhibition (Cousens et al., 1979) of the histone deacetylase enzymes (Sealy and Chalkley, 1978a; Candido et al., 1978; Vidali et al., 1978; Boffa et al., 1978). Hyperacetylation occurs within 24 hours after the addition of butyrate and is freely reversible.

Acetylation of the ϵ amino group of lysine residues in the amino terminus of the histones is thought to weaken the protein's interaction with the phosphate groups of the DNA. An alteration of the chromatin structure is indicated by an enhanced accessibility of the DNA to DNase I and micrococcal nuclease digestion (Vidali et al., 1978; Simpson, 1978; Nelson et al., 1978; Sealy and Chalkley, 1978b) and by an increased availability of histone H3 to phosphorylation by a nuclear Ca^{2+} -dependent protein kinase (Whitlock et al., 1980). Several groups have correlated enhanced nuclease digestion with potential gene activity (Weintraub and Groudine, 1976; Garel et al., 1977; Levy and Dixon, 1977; Nelson et al., 1978; Davie et al., 1978).

However, the role of histone hyperacetylation in transcription is unclear. While several reports correlate acetate incorporation into histones with mRNA synthesis (Allfrey et al., 1964; Pogo et al., 1968; Dobson and Ingram, 1980), other investigators observe no effect of histone hyperacetylation on the overall rate of in vitro transcription in isolated nuclei (Moore et al., 1978; Mathis et al., 1978; Lilley and Berendt, 1979). Perry and Chalkley (1982) report that histone acetylation increases the solubility of chromatin and that the complexity of the DNA associated with hyperacetylated nucleosomes is the same as the total DNA, indicating that hyperacetylation occurs over both active and inactive regions of the DNA. These observations, along with those of Reczek et al. (1982), suggest that butyrate may alter higher order chromatin structure (internucleosomal) rather than interactions

between DNA and core histones (intranucleosomal). Oliva and Mezquita (1982) and Grimes and Henderson (1983) suggest that hyperacetylation of H4 may correlate more closely to the displacement of nucleohistones by nucleoprotamine, which occurs during spermatogenesis, than to general transcriptional activity.

Prolonged exposure to butyrate impairs the ability of HTC cells to acetylate histones (Covault et al., 1982b). When butyrate is removed, the deacetylase enzymes recover more rapidly than the acetylase enzymes resulting in hypoacetylated histones. Utilizing these two acetylation states, Covault et al. (1982a) observed that histone acetylation has no immediate and direct effect on transcription in the intact cells. However, the rapid acetylation of a small population of histones may play a role in RNA synthesis initiation.

Butyrate also induces several other modifications of chromatin associated proteins. Boffa et al. (1981) examined the effect of butyrate on the acetylation, phosphorylation, poly (ADP)-ribosylation, and methylation of chromosomal proteins in HeLa cells. They report that butyrate inhibits the phosphorylation of histones H1 and H2A in a reversible, concentration, and time-dependent manner. Phosphorylation of non-histone proteins was stimulated, unaffected, or inhibited depending upon the protein examined. No effect was observed on the poly (ADP)-ribosylation of histones in cells treated with butyrate while methylation of histones and proteins associated with hnRNP particles is inhibited. Butyrate also leads to the dephosphorylation of histone H1

(D'Anna et al., 1980a) and an increased acetylation of high mobility group proteins 14, 17, 1, and 2 (Sternier et al., 1978; Sternier et al., 1979; Sternier et al., 1981).

Other Inducers of Ectopic Protein Synthesis

When Ghosh et al. (1977) reported the induction of hCG synthesis in HeLa cells by butyrate, they suggested that the induction may be related to the inhibition of cell growth and DNA synthesis. For example, both erythroid differentiation (Leder et al., 1975) and myoblast differentiation (Holtzer et al., 1972) are preceded by a cessation of cell growth. In fact, several groups have observed that certain compounds which inhibit DNA synthesis also induce hCG, PAP, and other proteins. These compounds include hydroxyurea (Ghosh and Cox, 1977; Ghosh et al., 1977; Chou and Robinson, 1977b), thymidine (Park, 1981), fluorodeoxyuridine (Park, 1981), bromodeoxyuridine (Koyama and Ono, 1970; Bulmer et al., 1976; Chou and Robinson, 1977a), iododeoxyuridine (Goz, 1974), cytosine arabinoside (Moecon et al., 1970; Chou and Robinson, 1977b; Ghosh et al., 1977; Deutsch et al., 1977), aphidicolin (Cox and Park, 1982), and phosphonoformic acid (Cox and Park, 1982).

Most of these compounds have a proposed mechanism of DNA synthesis inhibition. Hydroxyurea (Lewis and Wright, 1974; Yeh and Tessman, 1978) and thymidine (Richard, 1978; Morris and Fischer, 1963) inhibit ribonucleotide reductase. Fluorodeoxyuridine inhibits thymidylate synthetase (Friedkin, 1973; Danenberg, 1977). Bromodeoxyuridine

(Bischoff and Holtzer, 1970; Stellwagen and Tomkins, 1971; Djordjevic and Szybalski, 1960) and iododeoxyuridine (Goz and Walker, 1976) act as deoxynucleoside analogs and are incorporated into the DNA. Cytosine arabinoside may inhibit DNA synthesis by either incorporation into the DNA (Kufe et al., 1980; Chu and Fischer, 1965; Silagi, 1965) or inhibition of DNA polymerase α (Graham and Whitmore, 1970). Aphidicolin (Ikegami et al., 1978; Ohashi et al., 1978; Oguro et al., 1979) and phosphonoformic acid (Helgstrand et al., 1978; Reno et al., 1978; Cheng et al., 1981) inhibit DNA polymerase α activity. In the case of aphidicolin-resistant mutants, several studies suggest that a major site of action may involve deoxynucleoside metabolism rather than DNA polymerase α . Cells resistant to aphidicolin have expanded deoxynucleoside triphosphate pools, particularly deoxyadenosine, and an altered ribonucleotide reductase, but not a less sensitive DNA polymerase α (Sabourin et al., 1981; Ayusawa et al., 1981).

Other compounds such as actinomycin D, bleomycin, ethidium bromide, macromycin, and cesalin, which inhibit DNA synthesis as a result of intercalation or DNA strand scission, do not induce either hCG- α or PAP in HeLa cells (Park, 1981). In choriocarcinoma cells, Chou and Robinson (1977b) observe that three compounds which alter DNA structure, cytosine arabinoside, mitomycin C and phleomycin, also induce alkaline phosphatase activity. It appears, in general, that those compounds which alter either deoxynucleoside metabolism or are themselves deoxynucleoside analogs also induce hCG- α and PAP (Park, 1981). The

mechanism of induction of most of these compounds is unknown. This study examines the expression of hCG- α and PAP in HeLa cells to see if they are co-ordinately regulated and to better understand the action of various inducers and effectors. By studying factors which alter the expression of these genes, important regulatory elements may be elucidated.

MATERIALS AND METHODS

Methods

Cell culture

Various HeLa cell lines were grown in 75 cm² culture T-flasks in minimum essential medium (MEM) supplemented (MEMC) with 0.06% L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, 0.22% sodium bicarbonate, and 6-7.5% serum (fetal calf or calf). These lines included HeLa 2, 2.1, and 2.2 from the American Type Culture Collection and HeLa S-3 from Norm Cooper and Dr. Bernard Moss at the National Institutes of Health (NIH). Cells were fed with fresh MEMC every 2-3 days. When cells reached confluency, they were passaged by adding 5 ml of 0.01% trypsin in Hank's balanced salt solution with 0.02% EDTA and without CaCl₂ and MgSO₄. Ten ml of MEMC was then added to the trypsinized cell suspension and 5 ml of this diluted cell suspension was added to 10 ml of MEMC in another 75 cm² flask.

Induction protocol

Most induction experiments were performed in 25 cm² culture T-flasks. One ml of a diluted trypsinized cell suspension from a confluent 75 cm² culture was added to 4 ml of MEMC per 25 cm² flask (about 6×10^5 cells/ 25 cm² flask). Cells were grown to near confluency. The experiment was then started by feeding the cultures with 5 ml of fresh MEMC and adding the appropriate inducer and/or other effectors. After 3 days growth under the experimental conditions, the cells and media were harvested.

Harvesting of cells

Cells were harvested by scraping the cells into the growth medium of that flask with a rubber policeman. Cells (attached and detached) and cell debris were removed from the medium by centrifugation at 2,000 rpm (980 x g) x 10 min in an International centrifuge. The cell free supernatant was transferred to a test tube and frozen for assay of hCG- α at a later time. The cell pellet was washed twice with 1 ml of 50 mM Tris-HCl (pH 7.4), 0.9% (w/v) NaCl (Tris-saline). The cell pellet was then resuspended in 0.5 ml of Tris-saline and frozen.

Preparation of cell extracts

The frozen cell suspension was thawed, and deoxycholate was added to 0.5% (w/v). The cells were disrupted by sonication with a Branson Sonifier (microtip, output setting 6, 50% duty cycle, 2 min). Aliquots of this crude sonicate were then assayed for alkaline phosphatase activity and protein content.

Alkaline phosphatase assay

Alkaline phosphatase (EC 3.1.3.1) activity was assayed according to the procedure described by Edlow et al. (1975). The assay mixture contained cell sonicate (100 μ l or less), 0.75 M 2-amino-2-methyl-1-propanol (pH 10.7), and 8 mM p-nitrophenyl phosphate in a total volume of 1 ml. The samples were incubated in a 37^o C water bath until the color in the extract tubes developed significantly above the blank. Assays containing less than 2 mU of activity were linear for

up to 30 min. The reaction was terminated by the addition of 0.5 ml of 0.2 N NaOH and removal of the sample from the water bath. The absorbance of the samples was measured at $\lambda=400$ nm in a Gilford spectrophotometer. The amount of enzyme activity in the sonicates was determined from the equation: $(A_{400}/t)(1.5\text{ml/s.v.})(1/18.3) = \text{units of alkaline phosphatase activity/ml}$, where t is the time of incubation at 37°C , A_{400} is the absorbance at 400 nm, 1.5 ml is the total assay volume, s.v. is the sample volume, and 18.3 is the mM extinction coefficient for p-nitrophenol. One unit of activity was defined as the amount of enzyme which would catalyze the hydrolysis of 1 μmole of p-nitrophenylphosphate in 1 minute at pH 10.7 and 37°C .

Protein assay

The amount of cellular protein in each sample was determined from crude cell sonicates according to the procedure of Lowry et al. (1951). Bovine serum albumin (BSA) was used as the protein standard.

Radioimmunoassay for hCG- α

The alpha subunit of human chorionic gonadotropin (hCG- α) was assayed via a competition reaction with radiolabeled hCG- α for antisera raised to urinary hCG- α (anti- α). The hCG- α used for the standard curve and radiolabeling was prepared by Canfield and supplied by the NIH (CR-123). Antisera was prepared against hCG- α purified by Cox (1981a).

The alpha subunit was radiolabeled with ^{125}I using a lactoperoxidase method (Roth, 1975). The reaction mix consisted of 15

μl of 0.05 M sodium phosphate (pH 7.4), 5 μg of hCG- α , 10 μl of 0.4 M sodium acetate (pH 5.6), 2 μl of bovine lactoperoxidase at 2.7 mg/ml in 0.1 M sodium acetate (pH 5.6) ($A_{412}/A_{280} = 0.65$), and 2 μl of 30% H_2O_2 diluted 1/15,000 immediately before use (67 μl /1 liter H_2O). The reaction was started by the addition of 0.5 mCi of $\text{Na}[^{125}\text{I}]$ (sp. act. = 17 Ci/mg). After 60-90 seconds, the reaction was stopped by the addition of 100 μl of a solution consisting of 16% sucrose, 1% KI, and 0.02% sodium azide. Iodinated hCG- α was separated from unbound ^{125}I by chromatography over a G-100 column in 50 mM potassium phosphate (pH 7.4), 0.1% BSA, 0.02% NaN_3 buffer. One ml fractions were collected at a rate of about 0.2 ml/min. The column fractions were counted in a manual solid scintillation gamma counter and those fractions corresponding to the radiolabeled monomer of hCG- α with at least 10^6 cpm/fraction were stored at 4°C . Radiolabelling by this procedure routinely resulted in the incorporation of 40-60% of the ^{125}I into hCG- α with a specific activity of about 50 $\mu\text{Ci}/\mu\text{g}$.

Antisera was raised to hCG- α in rabbits as outlined by Park (1981). The assay consisted of a standard curve with unlabeled hCG- α (1 ng to 100 ng) and medium samples (50 μl to 1 ml). The volume was brought to a total of 1 ml with 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% BSA and 0.02% NaN_3 . To each tube was added 50,000 cpm of radiolabeled hCG- α and 10 μl of anti- α sera (diluted 1:50). The anti- α sera was diluted such that 10 μl /1 ml would precipitate 30% of the hCG- α present. This mixture was incubated for a minimum of 15 hours at room

temperature. Following this initial incubation, 30 μ l of goat anti-rabbit IgG was added to precipitate the anti- α complexes. This second incubation went for 1 hour at 37°C and then overnight at room temperature. Alternatively, 0.2 ml of 10% Staphylococcus aureus solution (prepared as described below) in 50 mM potassium phosphate (pH 7.4), 0.9% NaCl (PBS) was added and incubated for at least 30 minutes at room temperature. The precipitate was pelleted by centrifugation at 2,000 rpm for 10 min in a Beckman TJ-6 tabletop centrifuge. The supernatant was transferred to another tube and the pellet was washed with either 1 ml of PBS containing 0.1% Brij for the goat anti-rabbit pellets or 1 ml of PBS for the S. aureus pellets. The wash supernatant was combined with the first supernatant. The amount of radioactivity in both the supernatant and pellet was measured using either a manual solid scintillation gamma counter or an automatic gamma counter. The assay results were expressed as the percent of ^{125}I cpm in the pellet (i.e., $[\text{^{125}I cpm pellet} / (\text{^{125}I cpm pellet} + \text{^{125}I cpm super})] \times 100$). The amount of the hCG- α present in the experimental samples was calculated by comparing the corresponding percent cpm in the pellet with the percent cpm in the pellets of the standard curve.

Separation of Staphylococcus aureus-bound protein A

The Cowen I strain of Staphylococcus aureus (ATCC 12598) was obtained from Dr. Peter Patee in the Department of Microbiology at Iowa State University. Protocol for preparing S. aureus was obtained from E.D. Rosenblum, Department of Microbiology, Health Science Center,

University of Texas, Dallas, Texas. One hundred ml of 3% trypticase soy broth (TSB) in a 250 ml Erlenmeyer flask was inoculated from a slant of Cowan I stock culture and grown overnight at 37°C with shaking. Multiples of one liter of TSB in 2 liter Erlenmeyer flasks were inoculated with 10 ml of the overnight culture. These one liter cultures were shaken at 37°C overnight but not longer than 15 hours. The cells were harvested by centrifugation at 10,000 x g for 10 min. The cells from all the flasks were combined into a pre-weighed centrifuge bottle and washed twice with PBS. The cell pellet was resuspended (w/v) to 10% in PBS. Concentrated formaldehyde was added to 1.5% (4.05 ml of 37% formaldehyde/100 ml) and the mixture was stirred for 90 minutes at room temperature. Following pelleting of the cells, they were resuspended to 10% (w/v) in PBS and heated at 80°C for exactly 4 minutes in 100 ml aliquots in a 1 liter flask. The suspension was immediately cooled on ice. This heat treatment was repeated until the whole suspension was processed. Cells were pelleted by centrifugation at 10,000 x g for 10 minutes and washed once with PBS containing 0.02% NaN₃. Cells were transferred to a pre-weighed bottle, pelleted, and resuspended to 10% (w/v) in PBS containing NaN₃. This 10% solution of S. aureus was used to pellet the antigen-antibody complexes of the hCG-α RIA.

Measurement of DNA, RNA, and protein synthesis

HeLa cells were grown to near confluency in 24 well cluster plates. Cultures were fed with 1 ml of fresh MEMC and the experimental effectors

were added. At the appropriate times, 1 μ Ci of the appropriate radiolabeled precursor was added to each well and incubated for 4 hours at 37°C, 5% CO₂/95% air. The radiolabeled precursors used for these experiments were [³H] deoxyadenosine or [³H] thymidine for measuring DNA synthesis, [³H] uridine for measuring RNA synthesis, or ¹⁴C-labeled aminoacid mix for measuring protein synthesis. Cells were harvested by transferring the medium to 12 x 75 mm test tubes and adding 200 μ l of trypsin to each well. When the cells detached, they were added to their corresponding medium. The cells were pelleted and the media supernatant was removed and frozen for a later hCG- α assay. The cells were washed twice with 1 ml of Tris-saline, and the final cell pellet was resuspended in 1 ml of the same buffer. One ml of ice cold 10% trichloroacetic acid (TCA) was added to 0.5 ml of the cell suspension and left on ice for 30 min. The other 0.5 ml of the cell suspension was frozen for a protein determination. The TCA precipitated material was collected on either Millipore 0.45 μ m pore nitrocellulose filters or Whatman GF-C glass fiber filters and washed with ice cold 5% TCA. The filters were dried, placed in a toluene fluor (19 g 2,5-diphenyloxazole (PPO), 0.95 g 1,4-bis-(5-phenyloxazolyl)-benzene (POPOP)/ 3.8 liters of Toluene) and counted in a Beckman scintillation counter.

Deoxynucleoside triphosphate (dNTP) assay

The levels of dNTP in a sample were determined using E. coli DNA polymerase I, a template primer, and radiolabeled dNTP (Walters et al., 1973; Skoog, 1970; Lindberg and Skoog, 1970). DNA polymerase I

catalyzed the incorporation of deoxynucleotides onto a template primer. The extent of the reaction could be controlled by limiting the availability of a particular nucleotide. Utilizing the radiolabeled nucleotide precursor complementary to the limiting nucleotide, the reaction was monitored via the incorporation of the radiolabeled nucleotide into TCA insoluble material. That is, since equimolar amounts of complementary nucleotides are incorporated, the level of incorporation of the radiolabeled nucleotide into TCA insoluble material reflected the level of its unlabeled complementary nucleotide present in the sample.

The assay consisted of establishing a standard curve with known amounts of unlabeled dNTP (0.5 to 10 pmoles from 0.5 μ M stocks) to compare with samples with unknown levels of the nucleotide. The volume of the samples and standards was brought up to 40 μ l with 10 mM Tris-HCl (pH 7.4). With the samples on ice, 160 μ l of a premix consisting of 100 μ l of 80 mM Tris (pH 8.0) for dATP/dTTP assays or 100 μ l of 80 mM Tris (pH 8.6) for dCTP/dGTP assays, 10 μ l of 100 mM $MgCl_2$, 20 μ l of 5 μ M complementary [3H]-dNTP, 3.4 μ l of 500 μ g/ml poly (dA dT) template primer for dATP/dTTP assays or 3.4 μ l of 500 μ g/ml poly (dI dC) template primer for dCTP/dGTP assays, 20 μ l of 25 units/ml DNA polymerase I and 6.6 μ l of deionized distilled water was added to each sample. The samples were placed in a 37°C water bath for one half hour. The reaction was stopped by chilling the samples on ice and adding 0.1 ml of a mix containing 6.4 μ moles sodium pyrophosphate, 4 μ moles EDTA, and 1.5

mg/ml BSA. Macromolecular material was precipitated by adding 1 ml of ice cold 10% TCA and keeping the samples on ice for 15 min. The precipitates were collected on Whatman GF/C glass fiber filters which had been presoaked with 0.2 ml of 0.1 M sodium pyrophosphate. The filters were washed with 10 ml of 5% TCA, dried, placed in Toluene scintillation cocktail fluor, and counted in a Beckman scintillation counter.

E. coli DNA polymerase I was diluted from a 4,000 U/ml stock with 100 mM Tris-HCl (pH 8.3), 1 mM 1,4-dithiothreitol (DTT), and 0.5 mg/ml BSA to 25 U/ml. The template primers (poly (dA dT) and poly (dI dC)) were dissolved in 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl. Deoxynucleoside triphosphates were diluted with 10 mM Tris-HCl (pH 7.4) to 0.5 μ M. The radiolabeled nucleotides were diluted to a 5 μ M concentration with 10 mM Tris-HCl (pH 7.4) from stocks of 1 mCi/ml [3 H] dATP (17 Ci/mmol), [3 H] dTTP (44 Ci/mmol), [3 H] dCTP (30 Ci/mmol), and [3 H] dGTP (16 Ci/mmol). The template primers and unlabeled deoxynucleoside triphosphates were supplied by P-L Biochemical Company and the radiolabeled precursors were supplied by Amersham. Bethesda Research Laboratories (BRL) supplied the E. coli DNA polymerase I.

Determination of intracellular cAMP

In order to accurately determine the intracellular levels of cAMP, it is crucial that acid soluble extracts are prepared as quickly as possible. Therefore, an aspirator was used to remove the medium and 2 ml of ice cold 5% TCA was added immediately to the attached cells. The

cells were scraped into the TCA with a rubber policeman and transferred to a 12 x 75 mm test tube. The plate was rinsed with 1 ml of ice cold 5% TCA and added to the original extract. The TCA insoluble material was pelleted by centrifugation at $9,800 \times g$ for 10 min. The supernatant was transferred to a 10 ml extraction tube and extracted 4 times with 1.5 volumes of ether which had been acidified by extraction with two equal volumes of 1 N HCl. The ether was discarded, a stream of N_2 was gently bubbled through the extract to remove residual ether, and the sample was stored at $-20^{\circ}C$. The extract was later thawed, lyophilized, and refrozen ($-20^{\circ}C$). This lyophilized material was thawed and resuspended in 0.5 ml of 10 mM Tris-HCl (pH 7.4). A 150 μ l aliquot was removed, brought to 90 mM Tris with 1 M Tris-HCl (pH 7.5), and assayed for cAMP using a [3H] cAMP assay kit and protocol supplied by Diagnostic Products Corporation. The TCA insoluble material was solublized in 0.5 ml of 0.5 N NaOH and assayed for protein.

Partial purification of HeLa-c

Medium was collected from HeLa S-3 cells grown in spinner culture and concentrated ten fold in an Amicon concentrator/dialyzer. Technical grade dioxane was added to 50% (v/v) in 4 portions (15 min intervals) and mixed for one hour at room temperature. Dioxane insoluble material was removed by centrifugation. Following dialysis against water, the dioxane soluble protein was precipitated by adding solid ammonium sulfate to 90% (w/v). The ammonium sulfate precipitate was collected by centrifugation and resuspended in buffer appropriate for the column

chromatography of the next purification step (see Column chromatography section, below).

Desialylation of hCG- α

Partially purified hCG- α was desialylated according to a modified procedure of Dufau et al. (1972). The sample was dialyzed against water and 1 M sulfuric acid was added to 60 mM. The sample was heated at 80°C for 90 min. After the sample had cooled, it was neutralized with 1 M NaOH and then dialyzed against PBS.

Column chromatography

DEAE-Cellulose: The ammonium sulfate precipitate was resuspended in 50 to 100 ml of water and dialyzed against the equilibration buffer, 10 mM potassium phosphate (pH 8.0) with 0.02% (w/v) NaN_3 . The sample was applied to the column (1 liter) in a volume of 50 to 100 ml. Unbound material was eluted with one liter of equilibration buffer, and bound material was eluted with a 2 liter gradient of NaCl (0 to 0.3M) in equilibration buffer. Ten ml fractions were collected with the initial 20 to 25 fractions being collected at a slower flow rate to facilitate binding of protein to the column. For all column chromatography, bulk protein was determined from the A_{280} of each fraction and HeLa- α was detected by RIA.

Sephadex G-75 Superfine: The G-75 column was equilibrated in 0.1 M ammonium bicarbonate, 0.02% (w/v) NaN_3 . The ammonium sulfate pellet was resuspended in equilibration buffer. For comparison with placental

hCG- α , 200,000 cpm of iodinated placental alpha was co-chromatographed with the HeLa sample. The protein was eluted with 500 ml of equilibration buffer and collected in 2.5 ml fractions. Elution of the ^{125}I -labeled hCG- α was monitored by counting each fraction in a solid scintillation counter.

Lectin Chromatography: Partially purified HeLa- α was chromatographed on Concanavalin A-Sepharose or Ricin-agarose as described by Cox (1981c). Both columns were washed with high salt buffer (0.1 M sodium acetate (pH 6.0); 1 M NaCl; 1 mM MgCl_2 ; 1 mM CaCl_2 ; 1 mM MnCl_2 ; 0.02% (w/v) NaN_3), and then equilibrated with PBS + 0.02% (w/v) azide at room temperature. The HeLa sample was applied in 1 ml aliquots at 5-15 min intervals until the entire sample was absorbed on the column. The sample was left undisturbed on the column for 30 min. Unbound material was then eluted with PBS + 0.02% (w/v) azide in 1 ml fractions. Matrix bound material was eluted from Con A with 0.2 M D-alpha methylglucoside in PBS and from the ricin column with 0.2 M D-galactose in PBS.

Isolation of material for mRNA analysis

Cytoplasmic soluble material was isolated from HeLa cells as described by White and Bancroft (1982). Cells grown in 25 cm² flasks were scraped into 5 ml of PBS containing 0.02% (w/v) sodium azide, washed once with PBS, and then pelleted in a 1.5 ml Eppendorf tube. The cells were resuspended in 45 μl of ice cold 10 mM Tris-HCl (pH 7), 1 mM EDTA, and lysed by adding two 5 μl aliquots of 5% (v/v) Nonidet P-40

detergent 5 min apart with mixing on ice. Nuclei were pelleted by centrifugation in a Fisher microfuge for 2.5 min. Fifty microliters of the supernatant were transferred to a 1.5 ml Eppendorf tube containing 30 μ l of 20X SSC (1X SSC=0.15 M NaCl, 0.015 M sodium citrate (pH 7) and 20 μ l of 37% (v/v) formaldehyde. The sample was heated at 60°C for 15 min and then stored at -70°C. The nuclear pellets were stored frozen, then resuspended in 0.5 ml of Tris-saline and prepared for protein and alkaline phosphatase assays.

Cytoplasmic samples were thawed and heated at 60°C for at least 15 min, then serially diluted in a 96-well microtiter dish from 1:30 to 1:5 with 15X SSC to a final volume of 150 μ l. Twenty microliters of each sample were also diluted with 15X SSC to a final volume of 150 μ l and treated with RNase (10 μ g/ml) for at least 1 hour at 37°C. One hundred microliters of each dilution were applied with suction to a sheet of nitrocellulose (BA 85 (0.45 μ m) presoaked in 20X SSC) supported on a no. 470 paper utilizing a 96 hole manifold filtration apparatus. The filtration apparatus, nitrocellulose, and support paper were supplied by Schleicher and Schuell. The samples were baked onto the nitrocellulose by drying at 80°C in a vacuum oven for 90 min.

Growth and isolation of α -cDNA plasmid

DNA complementary to hCG- α mRNA was cloned into the plasmid pBR322 by Fiddes and Goodman (1979) and generously donated by Dr. J. Fiddes. Ten ml of L-broth (1% Bactotryptone, 0.5% Bactoyeast extract, 1% NaCl, pH 7.5). was inoculated with E. coli strain RRI (Amp^r, Tet^s) carrying

the hCG- α plasmid and placed in a 37°C shaker. Two hours after inoculation, freshly filtered ampicillin was added to a concentration of 20 μ g/ml. Following an overnight growth the culture was diluted 1:50 into 1 liter of L broth and grown with vigorous aeration at 37°C. Growth of the culture was monitored by removal of 1 ml and measuring the absorbance (light scattering) of the aliquot at 600 nm. When the culture reached an O.D.₆₀₀ of 1.0 (determined by extrapolation of the 1 ml readings), chloramphenicol was added to 184 μ g/ml. Cells were grown overnight with vigorous shaking. The cells were harvested by centrifugation at 8,000 rpm for 10 min in a JA 10 rotor (11,320 x g). The cells were resuspended in Tris-sucrose (50 mM Tris-HCl (pH 8.0), 20% (w/v) sucrose) to a final volume of 25 ml.

Three ml of freshly prepared lysozyme solution (10 mg/ml in 0.25 M Tris-HCl, pH 8.0) were added and gently swirled continuously for 5 min on ice. Three ml of 0.25 M EDTA (pH 8.0) were added. With gentle swirling, 30 ml of 0.2% (w/v) Triton X-100, 50 mM Tris-HCl (pH 8.0) and 25 mM EDTA (pH 8.0) were gradually added to lyse the cells. The solution was placed at room temperature for 15 min and stirred frequently. The cellular debris was pelleted by centrifugation at 15,000 rpm for 30 min at 5°C in a JA 20 rotor (27,200 x g). The cleared lysate was carefully decanted to a precooled graduated cylinder.

Nine grams of CsCl were added to 8.7 ml of the cleared lysate. The CsCl was dissolved by adding it very slowly and gently rocking the solution from end to end. The solution was refrigerated overnight

followed by the gentle rocking to dissolve any undissolved CsCl. The refractive index of the solution was adjusted to 1.394 by the addition of either CsCl or the lysis solution, and then 0.5 ml of 10 mg/ml ethidium bromide was added. The sample was centrifuged at 38,000 rpm at 15°C in a Beckman Ti 50 fixed angle rotor until two bands of DNA were visible upon illumination with a UV light source (at least 40 hours). The lower band, consisting of covalently closed circular plasmid DNA, and was collected by first discarding the material above the band. The plasmid DNA was then removed using a plastic Pasteur pipet in less than 0.5 ml aliquots starting at the bottom of the band. Collection was done in a dark room to avoid direct light which would damage the intercalated DNA.

Ethidium bromide was removed from the plasmid by extraction with 2 volumes of isopropanol equilibrated with 20X SSC. Extraction was complete when the DNA sample fluoresced blue instead of pink upon exposure to long wavelength UV light. Residual ethidium bromide was removed by dialysis against 2 liters of TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentration (A_{260}) and the purity (A_{260}/A_{280}) was determined before freezing the sample.

Nick translation

Nick translation of the α -cDNA plasmid followed a modified procedure of Rigby et al. (1977) supplied by Dr. Richard Maurer at the University of Iowa. The DNase solution was activated the day of use by diluting 20 μ l of DNase (1 mg/ml in 0.01 M HCl) with 180 μ l of

activating buffer (0.01 M Tris-HCl (pH 7.5); 5 mM MgCl₂; and 1 mg/ml BSA) and setting the solution on ice for 2 hr. Immediately before use, 5 μ l of the activated DNase was diluted with 2 ml of the activating buffer. The reaction was mixed on ice and consisted of the sequential addition of 2 μ l of 10X buffer (0.5 M Tris-HCl (pH 7.5); 0.05 M MgCl₂), 2 μ l of 10X dNTP (0.2 mM dATP, dGTP, dTTP), 0.1-0.5 μ g of DNA in 2 μ l, 100 pmoles of [α -³²P] dCTP (> 600 Ci/mole) in 14 μ l, and 2 μ l of DNase. The DNase reaction was incubated for 5 min at room temp. The sample was chilled for 15 min on ice. Ten units of DNA polymerase I (supplied by BRL) was added and incubated at 15°C for 2-5 hr. At the end of the reaction, 10 μ l of 0.1 M EDTA and 40 μ l of 0.4 M NaOH were added and then heated at 68°C for 5 min. The nick translated probe was separated from unincorporated [³²P] dCTP by chromatography over a 10 ml Sephadex G-50 column equilibrated with the elution buffer (TE, pH 8.0). Fractions of 0.5 ml were collected and the elution of ³²P-labeled material was monitored by Cerenkov counting. Those fractions corresponding to the nick translated probe were stored at 4°C. Specific activities of > 1 x 10⁸ cpm/ μ g of DNA were routinely obtained with the 5 hr DNA polymerase I incubation.

Filter hybridization

The baked nitrocellulose filter was placed into a heat sealable plastic bag (Seal-a-Meal, Sears-Roebuck Co.). The filter was prehybridized at 42°C for 8 hours in hybridization buffer (50% (v/v) deionized formamide; 5X SSC; 50 mM sodium phosphate (pH 6.5); 250 μ g/ml

salmon sperm DNA; 1X Denhardt's solution (50X = 1% (w/v) Ficoll; 1% (w/v) polyvinylpyrrolidone; 1% (w/v) BSA (Pentax Fraction V) (Maniatis et al., 1982)). The prehybridization mixture was removed and replaced with hybridization solution (50 μ l/cm²) consisting of ³²P-labeled α -pBR probe (15,000-50,000 cpm/cm²) in 4 parts hybridization buffer and 1 part 50% (w/v) dextran sulfate at 42°C for 24 hours. The salmon sperm DNA and the α -pBR probe were boiled for 5 minutes and added immediately to the hybridization solution. To minimize the formation of air bubbles on the nitrocellulose, both prehybridization and hybridization solutions were warmed to 42°C before addition to the filter and the air in the bag was removed before it was sealed. The filter was removed and washed four times (5 min each) in 2X SSC and 0.1% (w/v) SDS at room temp. The filter was then washed two times (15 min each) in 0.1 X SSC and 0.1% (w/v) SDS at 50°C. The filter was dried between 2 pieces of 3 MM paper, taped with the RNA side up to another piece of 3 MM paper, covered with Saran Wrap, and placed in a film cassette against a sheet of X-ray film (Kodak XAR-5) and intensifying screen. The film was exposed for 12 hours at -70°C.

RESULTS

Comparison of hCG- α and PAP production in HeLa cell lines

HeLa cell lines produced hCG- α and PAP at levels characteristic of that cell line (Table I). Relative to one another, CCL 2.1 was the lowest hCG- α producing cell line (139 ng/mg protein/72 hr) while CCL 2 was the highest producer (486 ng/mg protein/72 hr). CCL 2.2 and S-3 produced intermediate levels of hCG- α (352 and 318 ng/mg protein/72 hr, respectively). With regard to PAP, HeLa S-3 had the highest activity (7.5 mU/mg protein) and CCL 2 had the lowest activity (1.6 mU/mg protein). HeLa 2.1 and 2.2 were intermediate in PAP activity (3.4 and 2.7 mU/mg protein, respectively). Thus, α production and PAP activity were not correlated. That is, the cell line with the highest constitutive level of α production had the lowest level of PAP activity (CCL 2). The lowest α producer had an intermediate level of PAP (CCL 2.1). Also, those cell lines exhibiting the greatest difference in PAP activity (2 and S-3) had similar rates of hCG- α production. This observation that high hCG- α synthesis did not also correspond with high PAP activity in the same cell line suggests that these two proteins are not regulated in a co-ordinate fashion.

In these experiments comparing α and PAP expression among HeLa cell lines, as well as the protein induction experiments presented in the following sections, the level of α production and PAP activity varied from experiment to experiment. Although the reason for this variability

Table 1

PAP activity and hCG- α production in various HeLa cell lines

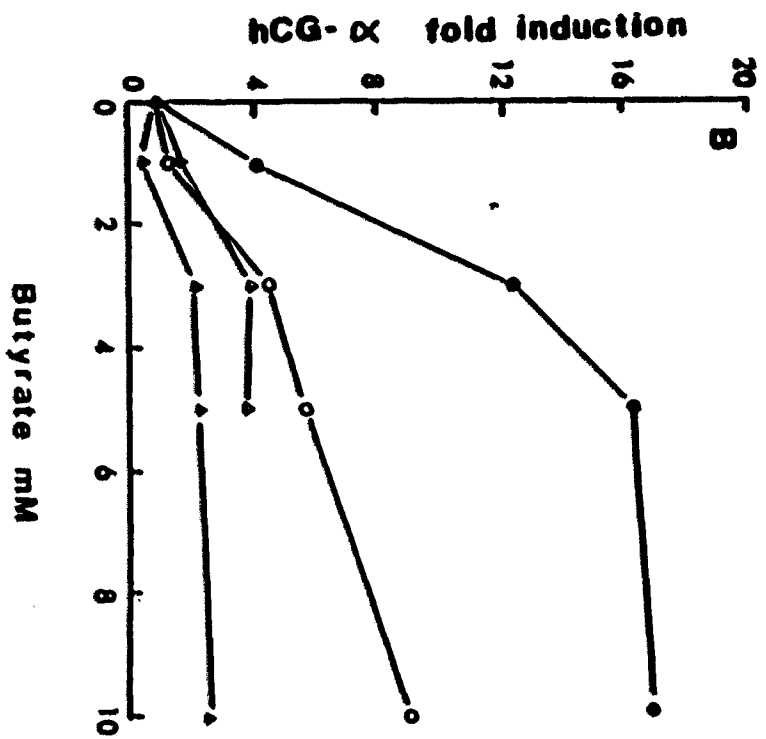
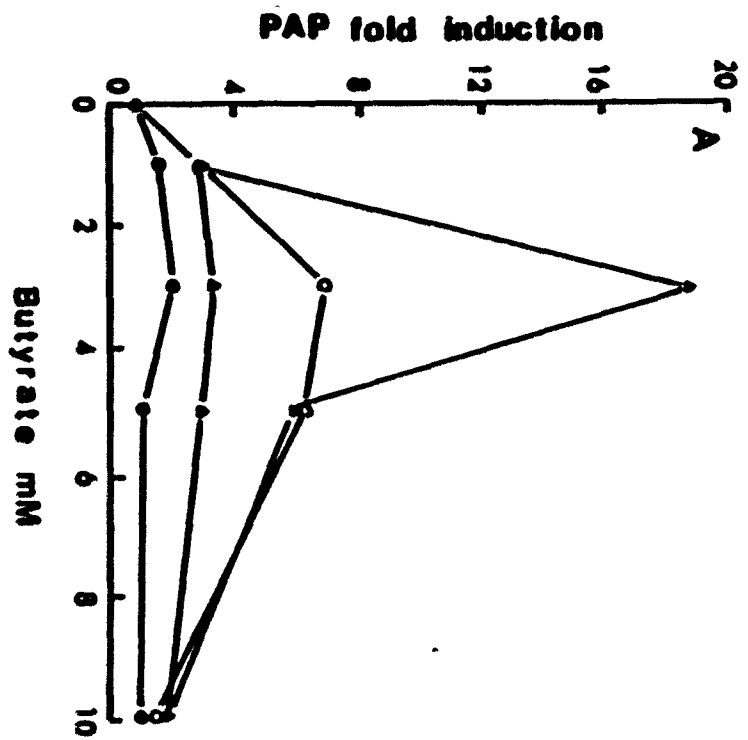
HeLa cell line	hCG- α ng/mg prot	PAP mU/mg prot
CCL 2	406 \pm 312 (8)	1.6 \pm 1.7 (8)
CCL 2.1	139 \pm 149 (9)	3.4 \pm 2.0 (7)
CCL 2.2	352 \pm 283 (8)	2.7 \pm 1.3 (8)
NIH S-3	318 \pm 229 (10)	7.5 \pm 6.1 (8)

^aVarious HeLa cell lines were grown for three days in MEMC, harvested, and assayed for hCG- α production and PAP activity as described in Materials and Methods. Certified cell lines (CCL) were provided by the American Type Culture Collection while NIH S-3 was a gift from Norm Cooper at NIH. The number of replicates for each determination is given in parenthesis.

is unclear, culture conditions, such as variability among batches of serum and cell density (see Fig 8) may significantly effect the expression of these genes. Thus, in cell line studies, results were compared between cultures grown simultaneously. While the magnitude of an effect on α and PAP expression may vary from one experiment to the next, the observations are valid as indicated by the variance illustrated in Table I and Table IV. A t-test analysis between the lowest and highest producers of both PAP (CCL 2 vs. NIH S-3) and hCG- α (CCL 2 vs. CCL 2.1) gave values of $t = 2.46$, $p \leq 0.014$ and $t = 2.79$, $p \leq 0.005$, respectively.

These cell lines also varied in their response to butyrate (Fig 1). For a given cell line, a strong stimulation of hCG- α production did not necessarily correlate with a strong induction of PAP. For example, HeLa S-3 showed the strongest induction of hCG- α but the lowest PAP induction. HeLa 2 had the lowest hCG- α induction but the highest PAP induction. Furthermore, the degree of induction did not correspond with the uninduced levels; that is, in this experiment, HeLa 2.1 and 2.2 differed by about 85 fold in basal levels of hCG- α but were similar in their response to butyrate. HeLa 2 and S-3, which had comparable control levels of hCG- α , exhibited about an 8-fold difference in response to butyrate. With regard to PAP activity, HeLa 2 had the lowest constitutive level but the greatest fold induction by butyrate. HeLa S-3 had the highest constitutive level of PAP but the weakest response to butyrate. These data suggest that butyrate may be

Figure 1. Dose-response curves for the butyrate induction of hCG- α and PAP in HeLa cell lines. [HeLa cells (CCL 2, 2.1, 2.2, and NIH S-3) were grown in MEMC (control) or in medium containing butyrate from 1 to 10 mM for three days. The cells were harvested and assayed for (A) PAP activity and (B) hCG- α as described in Materials and Methods. Control values are given in Table I. Symbols: 2 (Δ), 2.1 (\circ), 2.2 (Δ), S-3 (\bullet). Control values are 0.11, 0.54, 0.63, and 0.88 mU/mg protein of PAP and 50, 4, 341, and 71 ng α /mg protein for 2, 2.1, 2.2, and S-3, respectively.]



metabolized differently from one cell line to another, or that butyrate is metabolized similarly but that there are cell line differences in the regulation of hCG- α and PAP expression in response to butyrate. These data are also supportive of the discordant regulation of hCG- α and PAP.

Partial purification of HeLa hCG- α

The difference in alpha production between the various HeLa cell lines may be reflected by physicochemical properties of the protein. In order to characterize HeLa- α from a low and high producer (see next section), a scheme to partially purify alpha from the medium was developed using spinner cultures of S-3 cells as described in Materials and Methods. Fig 2 shows the chromatography of this material over a column of DEAE-cellulose. The majority of hCG- α did not bind to the column while the majority of protein did bind. The unbound material (fractions 70 to 160) was pooled and the protein was precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ to 90% (w/v). The pellet was collected by centrifugation at 25,860 x g for 30 min and then resuspended in 5 ml of 0.1 M NH_4HCO_3 . This material was chromatographed over a column of Sephadex G-75 superfine as described in Materials and Methods except radiolabeled hCG- α was not included. The chromatography profile shows that HeLa- α eluted in a volume of buffer greater than that of the majority of protein (Fig 3). Calculations in Table II indicate that a purification of about 5,000 fold was achieved by this protocol.

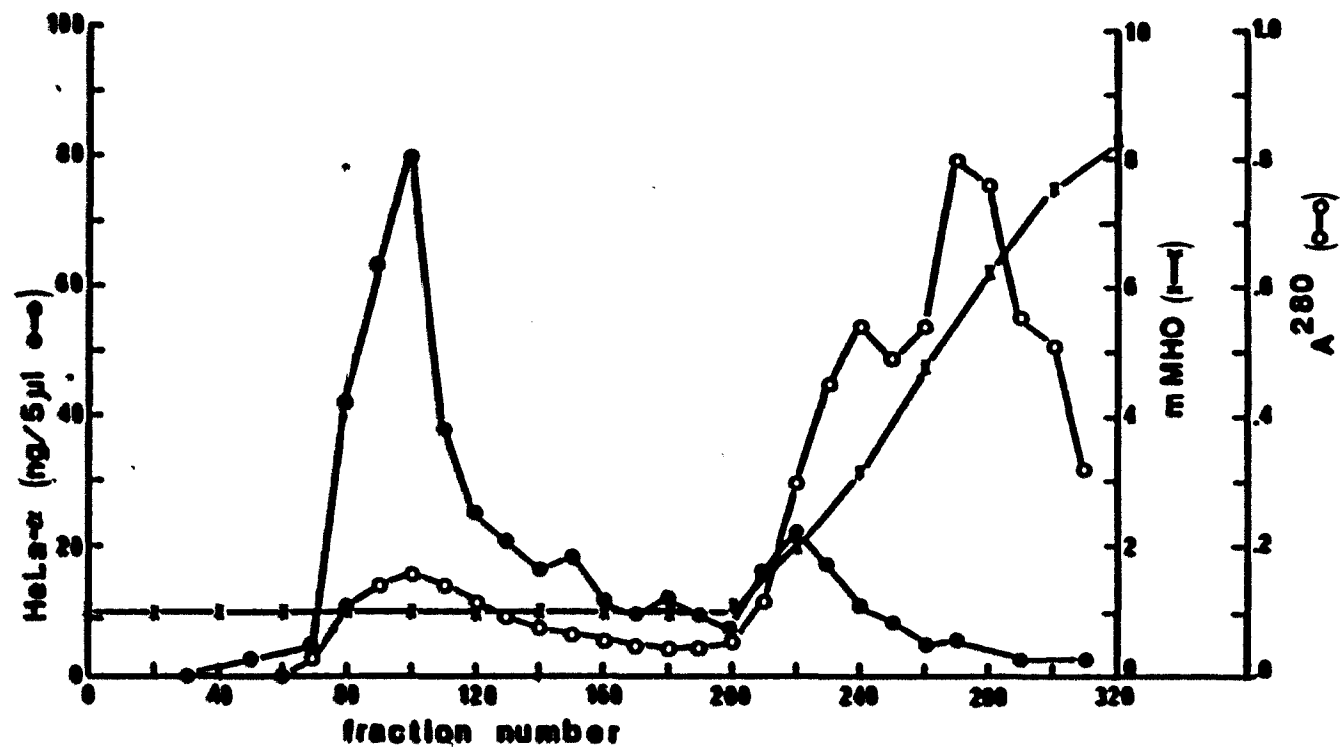


Figure 2. Chromatography of Hela-α on DEAE-cellulose. [Partially purified Hela-α was chromatographed over a column of DEAE-cellulose as described in Materials and Methods.]

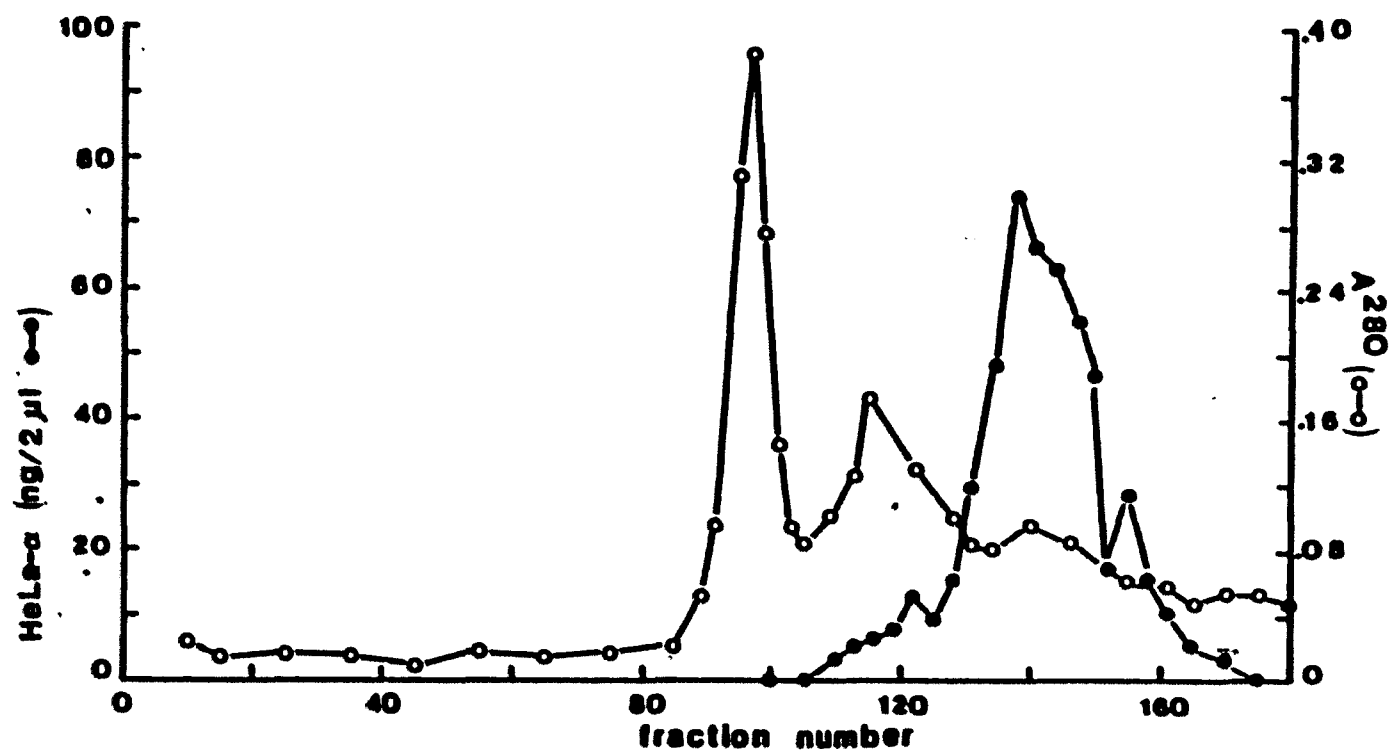


Figure 3. Chromatography of HeLa- α on Sephadex G-75 Superfine. [Partially purified HeLa- α was chromatographed over a column of Sephadex G-75 Superfine as described in Materials and Methods.]

Table II
Purification of HeLa-a ^a

Sample	HeLa-G μg	Protein A ₂₈₀	$\frac{\mu\text{g-G}}{A_{280}}$	Fold purification
Amicon concentrate	36,000	1.5×10^6	0.02	1
(NH ₄) ₂ SO ₄	3,300	1.6×10^3	2.03	102
Pooled G-75	598	6	99.67	4984

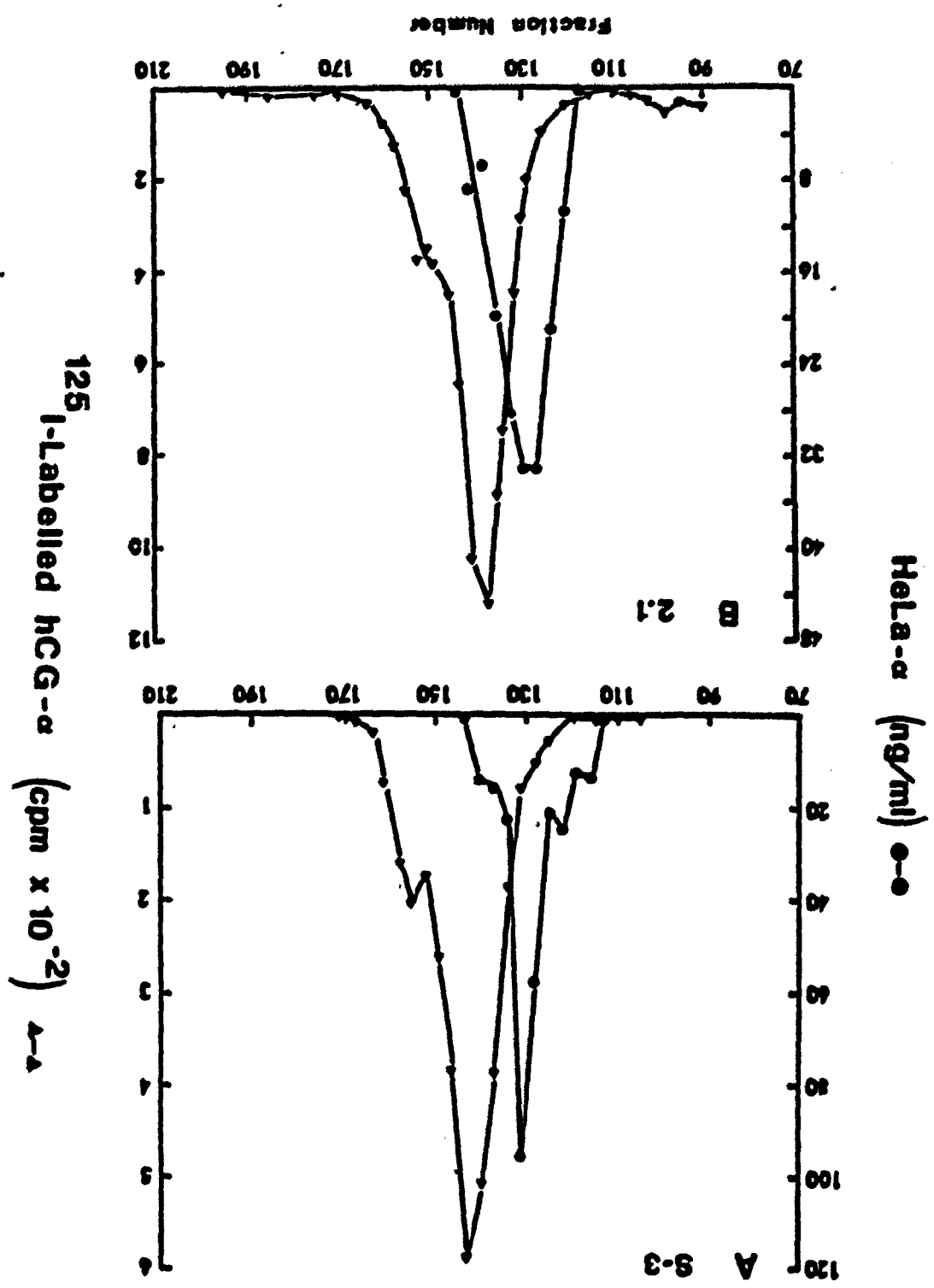
^aHeLa-G was partially purified as described in the text and in Materials and Methods.

Comparison of hCG- α from HeLa S-3 and 2.1

Lieblich et al. (1976) observed that hCG- α secreted by HeLa 2.2 cells had a lower elution volume on gel chromatography than hCG- α suggesting that the ectopic protein had a higher molecular weight than the normal subunit. Differences in the carbohydrate moiety of the two proteins may account for this chromatographic behavior (Cox 1981b). To see if the difference in alpha production by the various cell lines was due to differences in the synthetic pathway, the alpha protein synthesized by S-3 (a high producer) was compared with that of 2.1 (a low producer). Medium from 2.1 and S-3 cells was extracted with 50% dioxane (v/v), dialyzed against water, and precipitated with 90% $(\text{NH}_4)_2\text{SO}_4$ (w/v). The pellet was resuspended in 0.01 M NaHCO_3 , lyophilized to dryness, and resuspended in 0.1 M NH_4HCO_3 . These partially purified samples were co-chromatographed with ^{125}I -labeled urinary hCG- α over a column of Sephadex G-75 Superfine. The elution profiles show that the HeLa- α from both cell lines chromatographed similarly to each other and at a slightly higher apparent molecular weight than urinary hCG- α (Fig 4). Thus, there does not appear to be a significant difference in the overall size or shape of hCG- α in these two cell lines.

Differences in the ectopic production of hCG by various tumors has been suggested to be related to the level of protein glycosylation (Yoshimoto et al., 1979). Since no difference in the size of HeLa- α produced by 2.1 and S-3 cells was observed, the carbohydrate composition

Figure 4. G-75 column chromatography of HeLa- α and ^{125}I -labelled hCG- α . (HeLa- α was partially purified and co-chromatographed with ^{125}I -labelled authentic hCG- α as described in the text. Fractions of 2.5 ml were collected and assayed for HeLa- α by RIA. ^{125}I -labelled hCG- α was detected by solid scintillation spectroscopy. The amount of S-3 HeLa- α applied to the column was 2465 ng and recovery was 94%. 1300 ng of 2.1 HeLa- α was chromatographed and 89% was recovered. (A) S-3, (B) 2.1.]



of the two samples was examined by chromatography on lectin columns of Concanavalin A-Sepharose and Ricin-agarose. Con A binds D-glucose and D-mannose residues while Ricin has a specificity for D-galactose. Glycoproteins bind to these lectins because the carbohydrate structure of asparagine linked glycoproteins contain a high mannose core and terminal galactose residues. Medium from 2.1 and S-3 cultures was extracted with 50% dioxane (v/v) and precipitated with 90% $(\text{NH}_4)_2\text{SO}_4$ (w/v). The pellets were resuspended and chromatographed over Concanavalin A-Sepharose as described in Materials and Methods. The Con A elution profiles of these samples show that the majority of the alpha subunit synthesized by both cell lines bound to Con A, indicating that they are glycosylated (Fig 5). The peak of the S-3 bound material eluted in 18 ml of 0.2 M D- α -methylglucoside while the peak of the 2.1 bound material eluted in 13 ml. The Con A-bound material (2.1 fractions 65-87 or S-3 fractions 42-58) was pooled, dialyzed against water, and lyophilized to dryness. The lyophilized material was resuspended in water, desialylated (to expose the penultimate galactose residues of the carbohydrate side chains and thereby facilitate binding to ricin (Eagon and Heath, 1977), and chromatographed on Ricin-agarose as described in Materials and Methods (Fig 6). The similar chromatography of both samples suggested that the HeLa- α synthesized by both cell lines were similar in their terminal glycosylation composition. From these lectin binding data, it was inferred that the difference in hCG- α production between 2.1 and S-3 does not result from a gross difference in glycosylation.

Figure 5. Concanavalin A Sepharose chromatography of hCG- α from HeLa S-3 and 2.1 cell lines. [HeLa- α was partially purified from the medium of S-3 and 2.1 cells and chromatographed on Concanavalin A Sepharose as described in Materials and Methods. One ml fractions were collected and assayed for hCG- α by RIA for the 2.1 sample while 2 ml fractions were collected for the S-3 sample. The arrows denote elution with 0.2 M α -methyl glucoside. The amount of HeLa- α applied was 3 μ g of S-3 α and 3.1 μ g of 2.1 α . Recoveries were 91% and 89% for S-3 and 2.1, respectively. (A) Elution profile for S-3 HeLa- α . (B) Elution profile for 2.1 HeLa- α .]

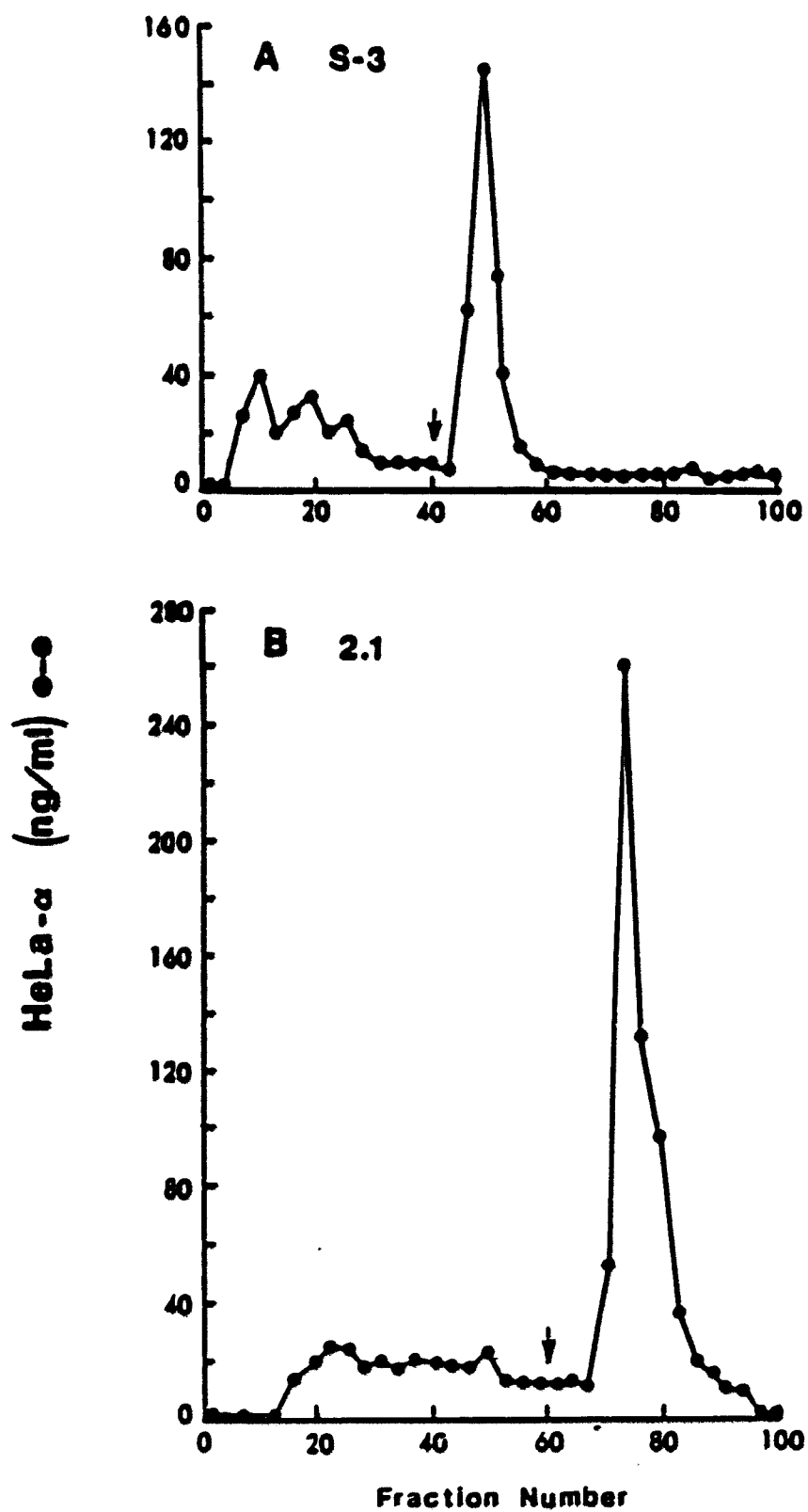
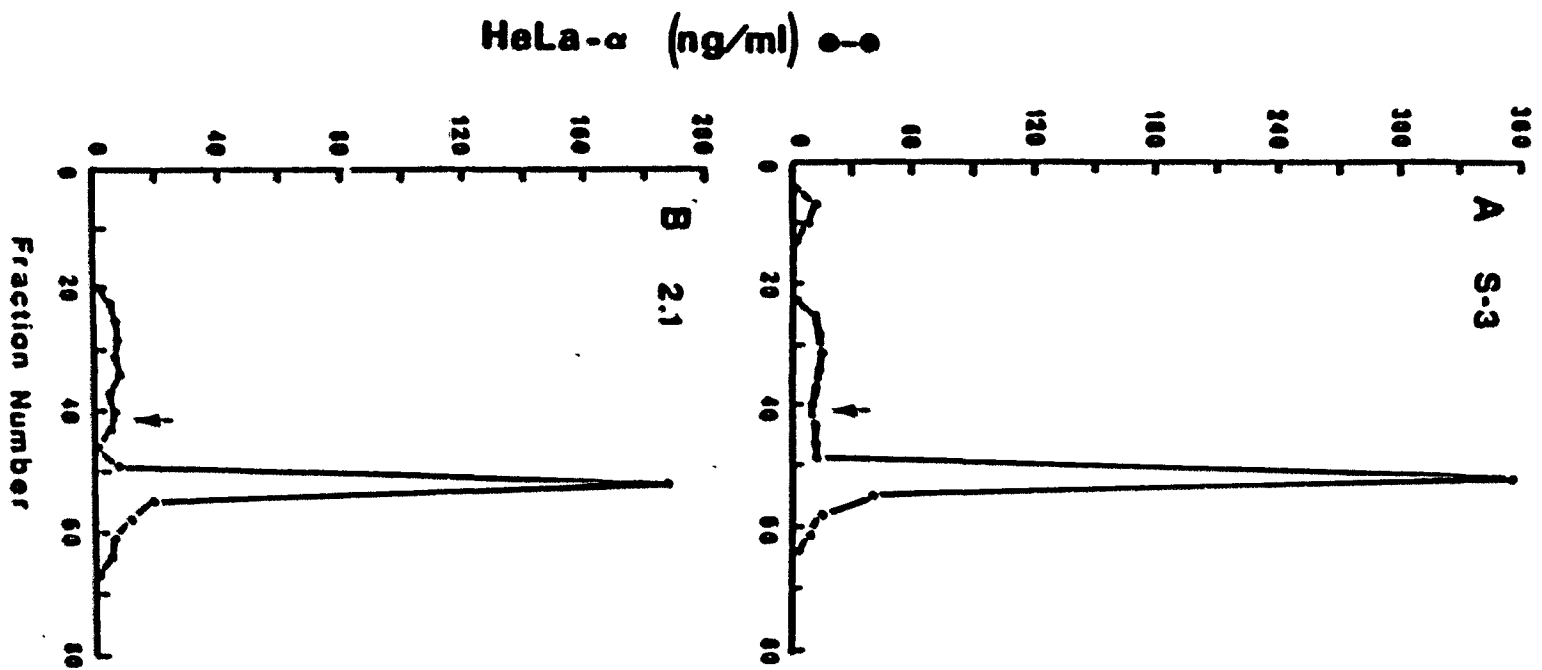


Figure 6. Ricin agarose chromatography of desialylated hCG- α from HeLa S-3 and 2.1 medium. [The Con A bound HeLa- α from Fig 5 was pooled, desialylated, and chromatographed over a column of Ricin agarose as described in Materials and Methods. Arrows indicate the elution with 0.2 M D-galactose. Of the 1.6 μ g of S-3 HeLa- α and the 1.0 μ g of 2.1 HeLa- α applied to the column, about 97% and 80%, respectively were recovered. (A) HeLa S-3 (B) HeLa 2.1.]



Induction of hCG- α and PAP

An alternative approach to examining the regulation of alpha and alkaline phosphatase production involved the use of agents which modified their expression.

Determination of assay and culture conditions for PAP activity and hCG- α induction

Since placental alkaline phosphatase is a membrane bound protein, the assay may depend upon the method of cell extract preparation. Addition of deoxycholate (0.2-0.5%) to the cell suspension during sonication enhanced the measurement of alkaline phosphatase activity about 2.5 fold (Fig 7). Equivalent levels of alkaline phosphatase activity in the crude sonicate and in the post centrifugation supernatant indicated that PAP was efficiently extracted from the plasma membrane by deoxycholate. From these data, it was decided to include 0.5% deoxycholate during sonication and to assay the activity in the crude sonicate.

Production of hCG- α by HeLa cells varied with the cell density (Fig 8). In control cells, production was higher at low cell densities (2×10^5 cells/25 cm²), but in response to 3 mM butyrate, subunit synthesis was stronger at high cell densities (1×10^7 cells/25 cm²). To optimize induction conditions, most experiments were performed with cultures at high cell densities.

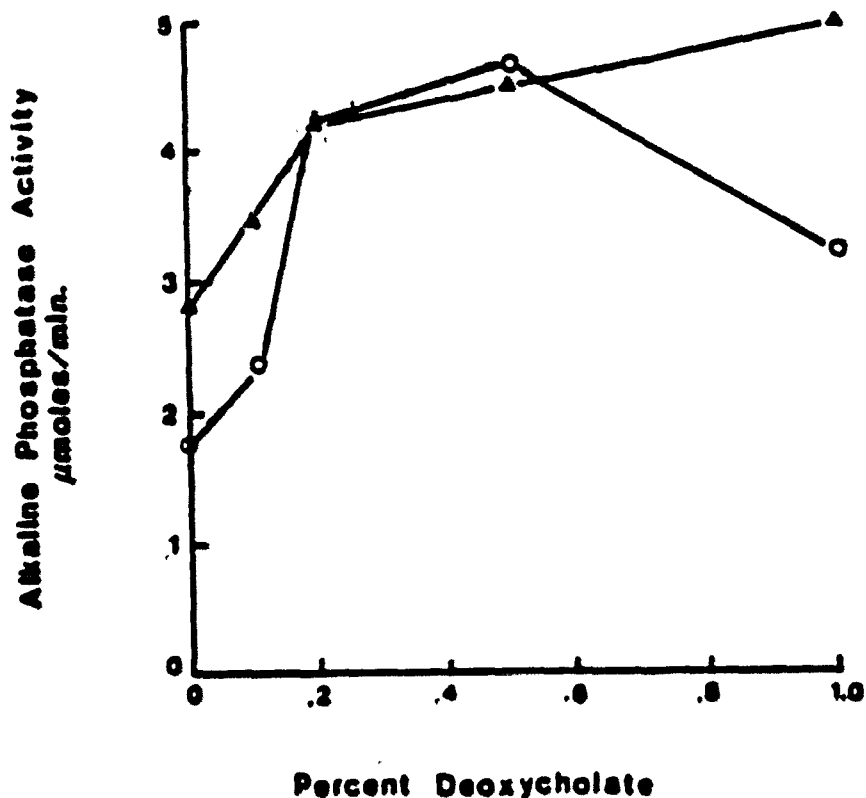
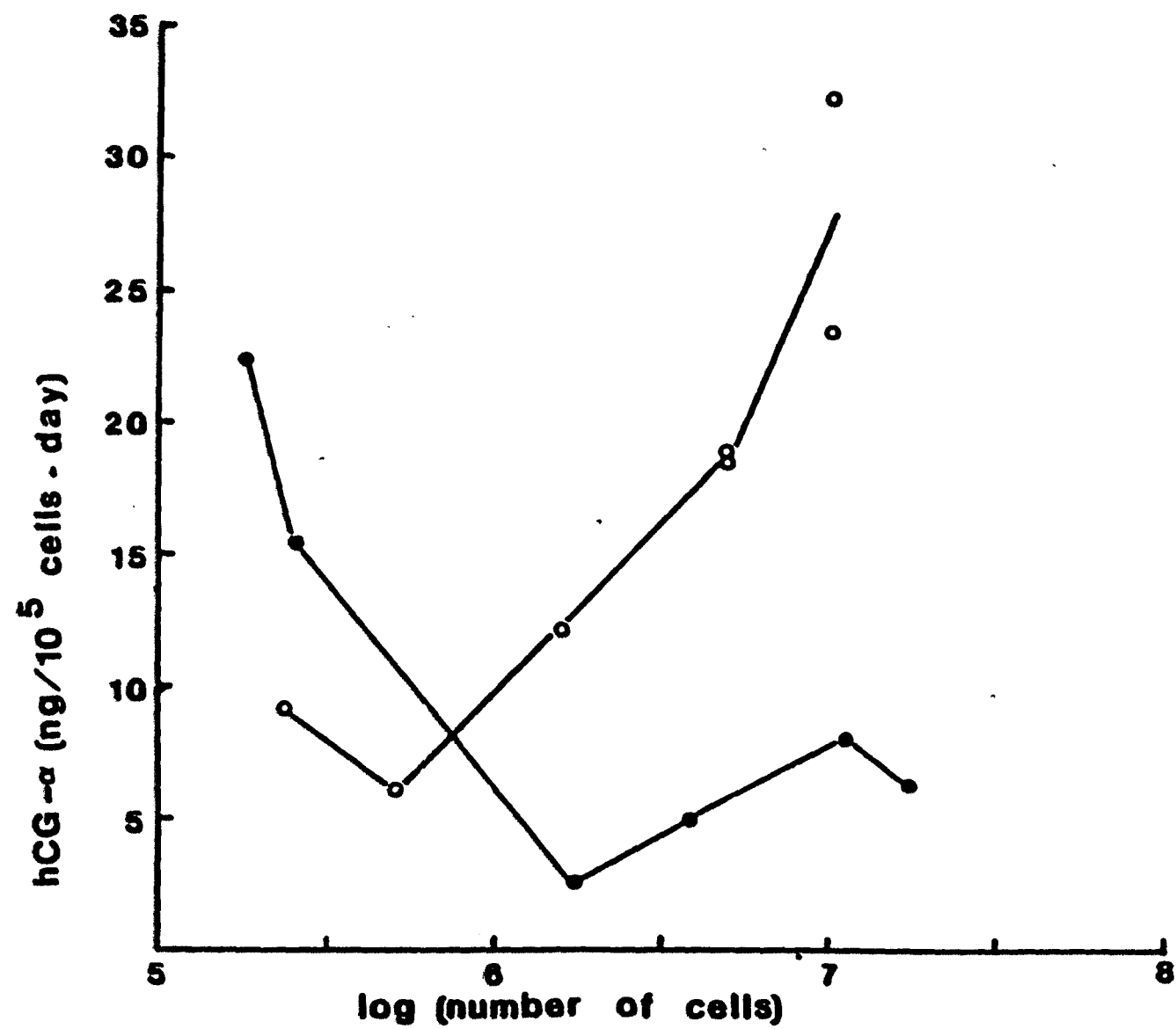


Figure 7. Determination of sonication and assay conditions for PAP activity in cell extracts. [Several flasks of confluent HeLa S-3 cultures were harvested, pooled, and divided into 0.8 ml aliquots. These samples were sonicated on ice in the presence of deoxycholate (DOC) from 0-1.0% (w/v). Alkaline phosphatase activity was assayed from either the crude sonicate or from sonicate cleared of particulate matter by centrifugation at 27,000 x g for 20 min. Symbols: pre-centrifugation (Δ), post-centrifugation (O).]

Figure 8. Synthesis and secretion of hCG- α as a function of cell density. [HeLa S-3 cells were plated at a density of 1×10^5 cells/25 cm² flask. One set of flasks (control) were fed daily with fresh MEMC and duplicate cultures were harvested every other day. A second set of flasks (butyrate) were fed every other day with fresh MEMC. Sodium butyrate (2 mM) was added to duplicate cultures in this second set and harvested 2 days later. Cell numbers were determined using a hemacytometer and hCG- α was assayed in the culture medium by RIA. Symbols: control (●), butyrate (○).]



Heat inactivation of HeLa alkaline phosphatase

As described in the Introduction, there are at least three different alkaline phosphatase isozymes in the human genome (Mulivor et al., 1978c; Badger and Sussman, 1976; McKenna et al., 1979). Although the alkaline phosphatase in HeLa cells has been characterized to be predominately the placental isozyme (which is heat stable), heat labile alkaline phosphatase activity has also been reported (Benham et al., 1978; Benham and Harris, 1979). It was of interest, therefore, to determine if the predominant isozyme in induced cells was the same as that in control cells. From the heat inactivation profiles presented in Fig 9, it appears that butyrate, BrdUrd, and cytosine arabinoside enhanced the activity of the major isozyme which is active in untreated cells. This isozyme had a heat inactivation profile similar to authentic PAP. Inhibition of the activity to 50% occurred at 71°C for PAP and HeLa control cells, 72°C for butyrate and BrdUrd treated cells, and 73°C for cytosine arabinoside induced activity. Since other isozymes of alkaline phosphatase are inactivated by heating at 65°C for 30 min, a mixture of isozymes would be expected to result in a bi- or multi-phasic inactivation curve.

Effect of butyrate concentration on α and PAP induction

One metabolite which greatly enhanced the production of hCG- α and PAP activity in HeLa cells was sodium butyrate. The effect of butyrate from 1 to 20 mM on the expression of hCG- α and PAP is illustrated in Fig 10. PAP activity increased proportionally to the butyrate concentration

Figure 9. Heat inactivation profiles of alkaline phosphatase activity from HeLa cell cultures. [HeLa S-3 cells were grown for 3 days in MEMC alone (control) or in medium containing 2 mM butyrate (Btr), 10 mM cytosine arabinoside (Ara-C), or 10 µg/ml 5-bromodeoxyuridine (BrdUrd). Cells were harvested and sonicated in 1 ml of Tris-saline buffer containing 0.5% DOC. Insoluble material was removed by centrifugation at 27,000 x g for 20 min. The supernatant was divided into 0.1 ml aliquots, heated for 5 min at 37° to 80° C, cooled on ice, and assayed for alkaline phosphatase activity. The activity of each heated sample is expressed as the percent activity compared to an unheated duplicate. Heat inactivation profiles were compared to that of placental alkaline phosphatase which was obtained from Miles Laboratories (15 U/mg). In this experiment, butyrate induced alkaline phosphatase about 3.6 fold, Ara-C about 2.4 fold, and BrdUrd about 4.8 fold.]

Alkaline Phosphatase Activity

% of unheated aliquot

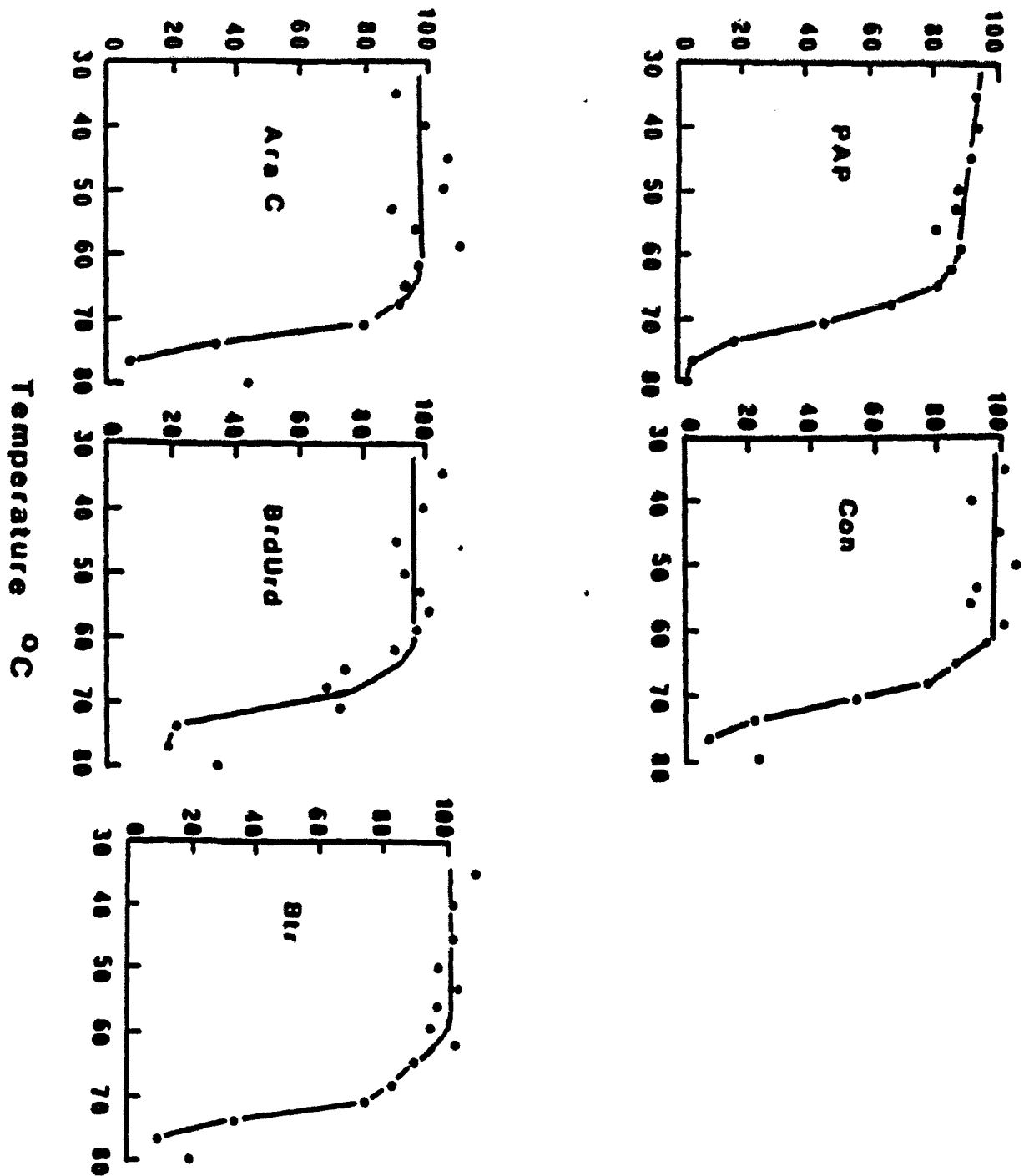
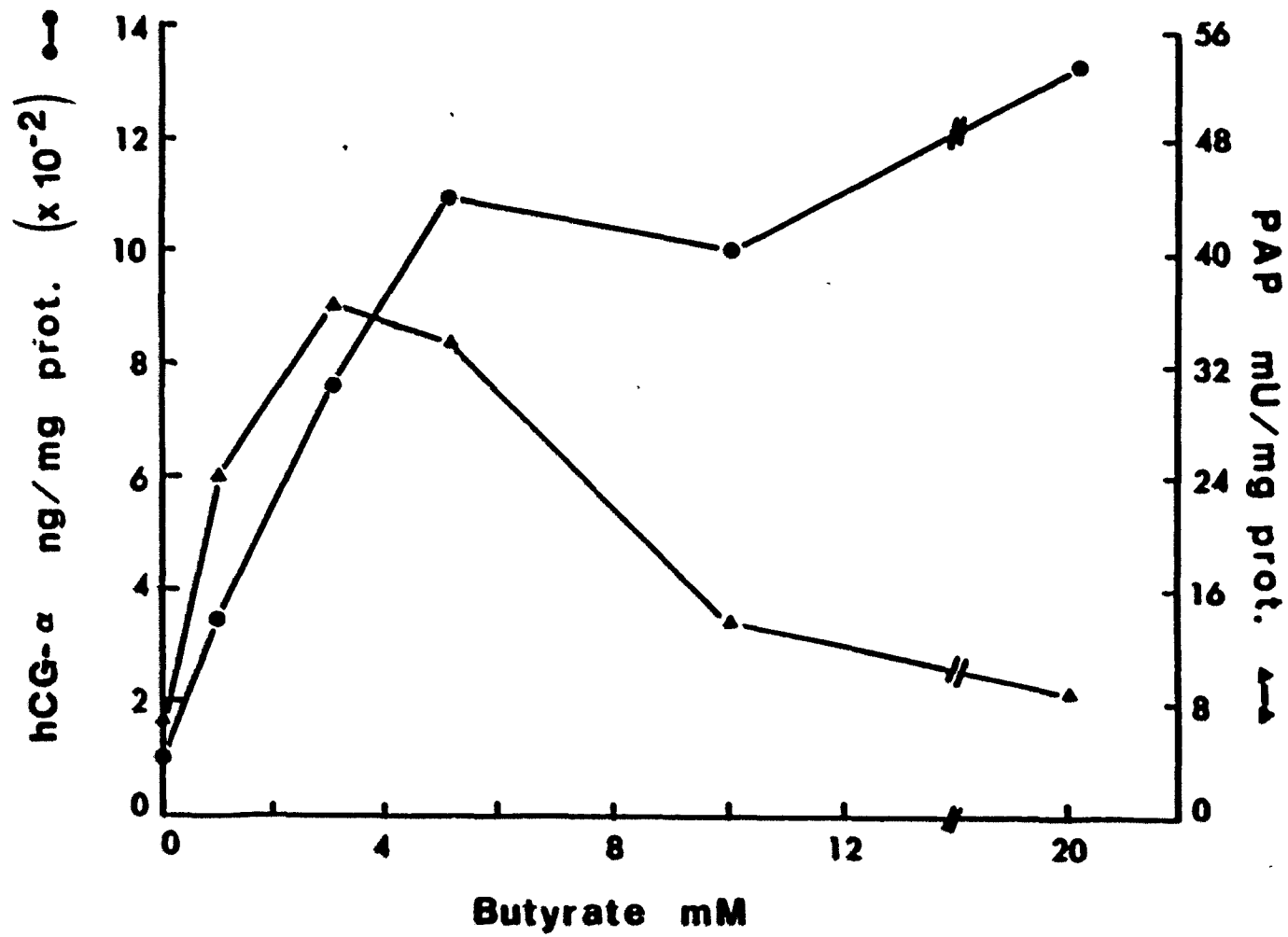


Figure 10. Induction of hCG- α and PAP by sodium butyrate. [HeLa S-3 cells were cultured to near confluency in 25 cm² flasks. They were then fed with fresh MEMC containing 0 to 20 mM sodium butyrate. After 72 hr, the cells were harvested and assayed for PAP activity and hCG- α as described in Materials and Methods. Symbols: hCG- α (●), PAP (▲).]

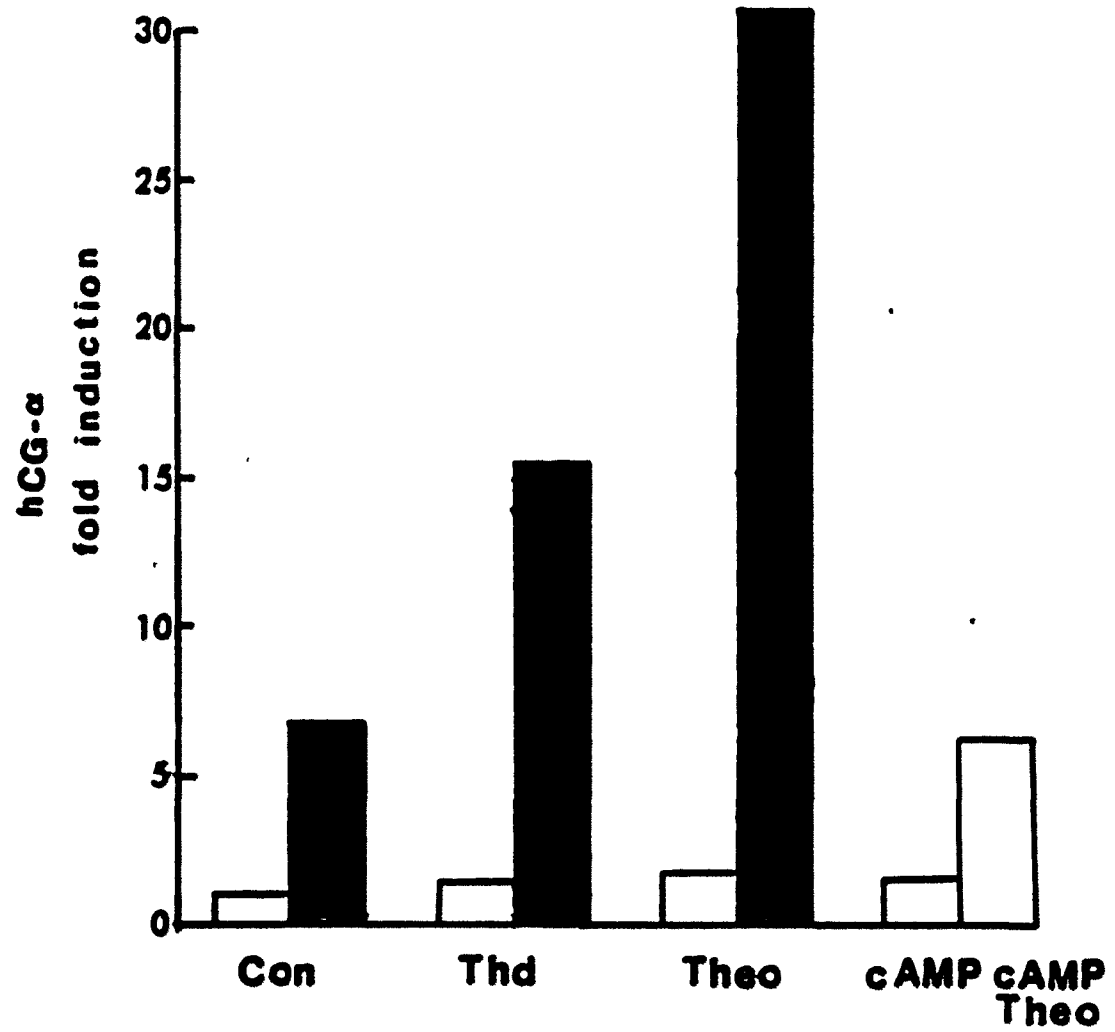


up to 3 mM, while higher concentrations were not as effective. The production of hCG- α was also proportional to the butyrate concentration with maximum induction occurring between 5 and 10 mM. This observation, that maximum induction of PAP occurs at a butyrate concentration lower than that required for maximum hCG- α production, is consistent with the idea that the expression of these two genes is not co-ordinately regulated.

Synergistic induction of hCG- α

In previous reports, the production of hCG and hCG- α was enhanced by various potential modulators of cAMP levels in normal placental cells and in human malignant trophoblasts but not in three non-trophoblastic cell lines, ElCo, CaSki, and DoT (Hussa et al., 1977, 1978). HeLa cells were not examined. Thus, it was of interest to investigate the possible involvement of cAMP in the synergism between butyrate and some of the inducers surveyed by Park (1981). In this regard, theophylline, thymidine, and cAMP were studied for their effect on induction by butyrate. Pronounced increases were observed with theophylline and thymidine (Fig 11). Butyrate (3 mM) alone induced hCG- α about 6.8 fold, thymidine (300 μ M) about 1.4 fold, and theophylline (1 mM) about 1.8 fold. In other experiments, theophylline occasionally induced hCG- α comparable to butyrate alone; the reason for this is not known. However, the combination of butyrate and thymidine resulted in a 15.5 fold induction of hCG- α , while butyrate and theophylline resulted in a 30.8 fold induction. PAP activity was induced slightly by butyrate or

Figure 11. Effect of thymidine and theophylline on the butyrate-mediated induction of hCG- α and PAP. [HeLa S-3 cells were grown in the presence of 3 mM butyrate, 500 μ M thymidine, 1 mM theophylline, and 1 mM cAMP according to the induction protocol in Material and Methods. (A) The concentration of hCG- α in control cultures was 236 ng/mg protein. (B) PAP activity in control cultures was 30.6 mU/mg protein. Cultures treated with butyrate are denoted by the solid bars.]



thymidine alone, while theophylline appeared to inhibit PAP activity. Synergism was not observed with the butyrate and thymidine combination or the butyrate and theophylline combination. Cyclic AMP (cAMP) did not induce either protein. The combination of cAMP and theophylline induced hCG- α only 6.2 fold and did not enhance PAP activity.

Although the methylxanthines caffeine and theobromine are also known to increase cAMP concentrations, synergism with butyrate was specific for theophylline. Table III shows that 1 mM theophylline, but not 1 mM xanthine, 1 mM caffeine, nor 1 mM theobromine, was synergistic with butyrate for the induction of hCG- α . All of the xanthine compounds inhibited the butyrate induction of PAP. These data suggest that theophylline mediates its synergistic effect with butyrate by a mechanism other than an increase in cAMP levels. Also, the synergistic induction of hCG- α and the inhibition of PAP induction suggests discordant regulation of these two proteins.

The time course of hCG- α accumulation by cells treated with thymidine and theophylline alone or in combination with butyrate is presented in Fig 12. Alpha levels did not increase over control values until 48-72 h after the addition of Thd to the medium. In this experiment, as well as most others, the induction of α by the combination of Thd and Btr was additive or slightly synergistic. Theophylline induced hCG- α to approximately the same extent as butyrate for at least 48 hours. In contrast, cells treated with butyrate and theophylline exhibited α levels increased relative to control after only

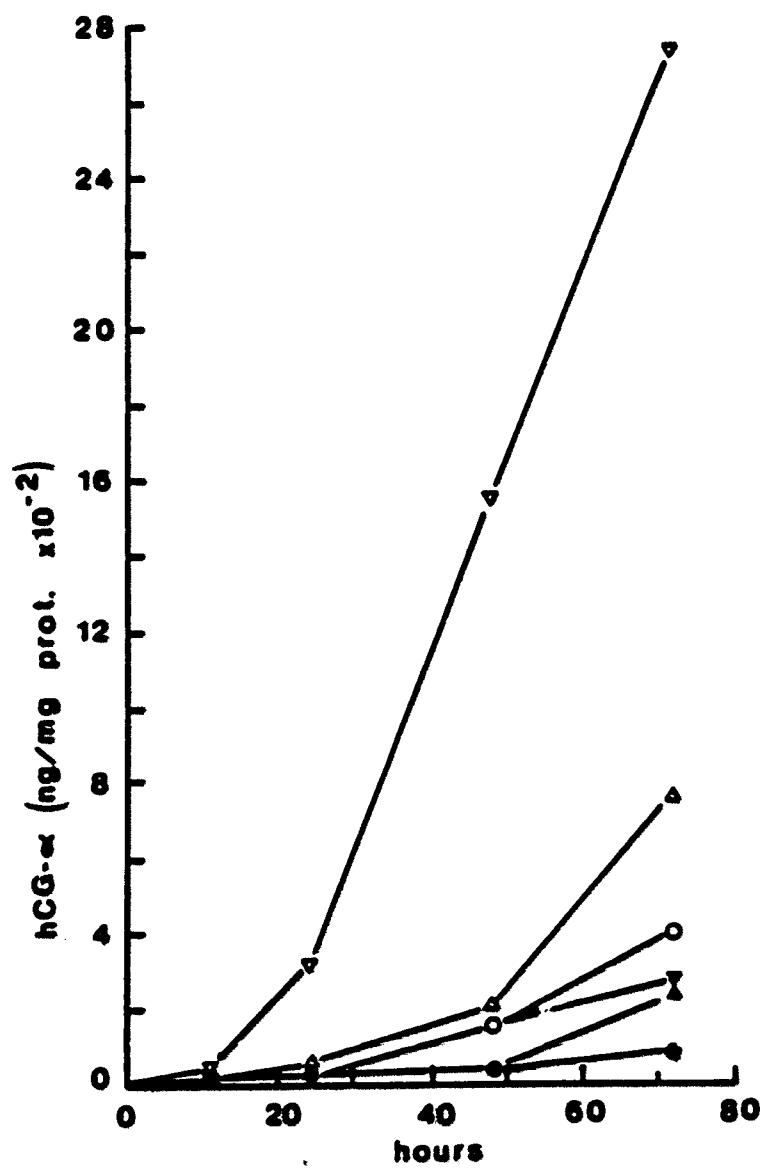
Table III

Effect of xanthine and xanthine derivatives on the butyrate
induction of hCG- α and PAP^a

Sample	hCG- α		Fold induction	PAP		Fold induction
	Values ng/mg protein	Mean		Values mU/mg protein	Mean	
Control	0.58, 0.68	0.63	1.0	0.15, 0.17	0.16	1.0
Xanthine	0.58, 0.26	0.42	0.7	0.15, 0.13	0.14	0.8
Caffeine	0.99, 0.88	0.94	1.5	0.09, 0.10	0.10	0.6
Theobromine	0.85, 0.51	0.68	1.1	0.11, 0.10	0.11	0.6
Theophylline	1.3, 1.2	1.2	2.0	0.11, 0.10	0.11	0.6
Butyrate	4.5, 2.7	3.6	5.8	8.4, 5.7	7.1	48.8
Butyrate Xanthine	1.8, 3.8	2.8	4.4	2.7, 3.8	3.2	20.2
Butyrate Caffeine	4.7, 4.8	4.7	7.5	1.8, 2.3	2.0	12.8
Butyrate Theobromine	6.1, 4.9	5.5	8.8	2.7, 2.8	2.8	17.4
Butyrate Theophylline	13, 19	16	25.4	2.2, 2.1	2.2	13.6

^aHeLa 2.2 cells in 25 cm² flasks were treated with 1 mM xanthine, 1 mM caffeine, 1 mM theobromine, or 1 mM theophylline alone or in combination with 3 mM butyrate for 3 days. Cells were harvested and assayed for hCG- α as described in Materials and Methods. PAP activity was assayed from either 100 μ l or 50 μ l of the cell sonicate in a total volume of 1 ml containing 0.75 M 2-amino-2-methyl-1-propanol, pH 10.7, and 8 mM p-nitrophenylphosphate. The reaction was monitored by measuring the A₄₀₀ in a Beckman 2400 DU Spectrophotometer using a Gilford 6051 chart recorder and the automatic cuvette changer. The cuvette chamber temperature was maintained at 37°C by means of a circulating water bath. The mM extinction coefficient of p-nitrophenol was determined to be 18. Assays of duplicate cultures were within 1% to 40% of the mean.

Figure 12. Time course of hCG- α accumulation in response to thymidine, theophylline, and butyrate. [HeLa 2.2 cells in 75 cm² flasks at near confluency were grown in 2 mM thymidine, 1 mM theophylline, or 3 mM butyrate as indicated below. At 12, 24, or 48 h after the addition of effectors, 1 ml aliquots were removed from the medium and analyzed by RIA. Cells were harvested after 72 h for protein determination. Symbols: control (●), Thd (▲), theophylline (▼), Btr (○), Btr + Thd (△), Btr + theophylline (▽).]



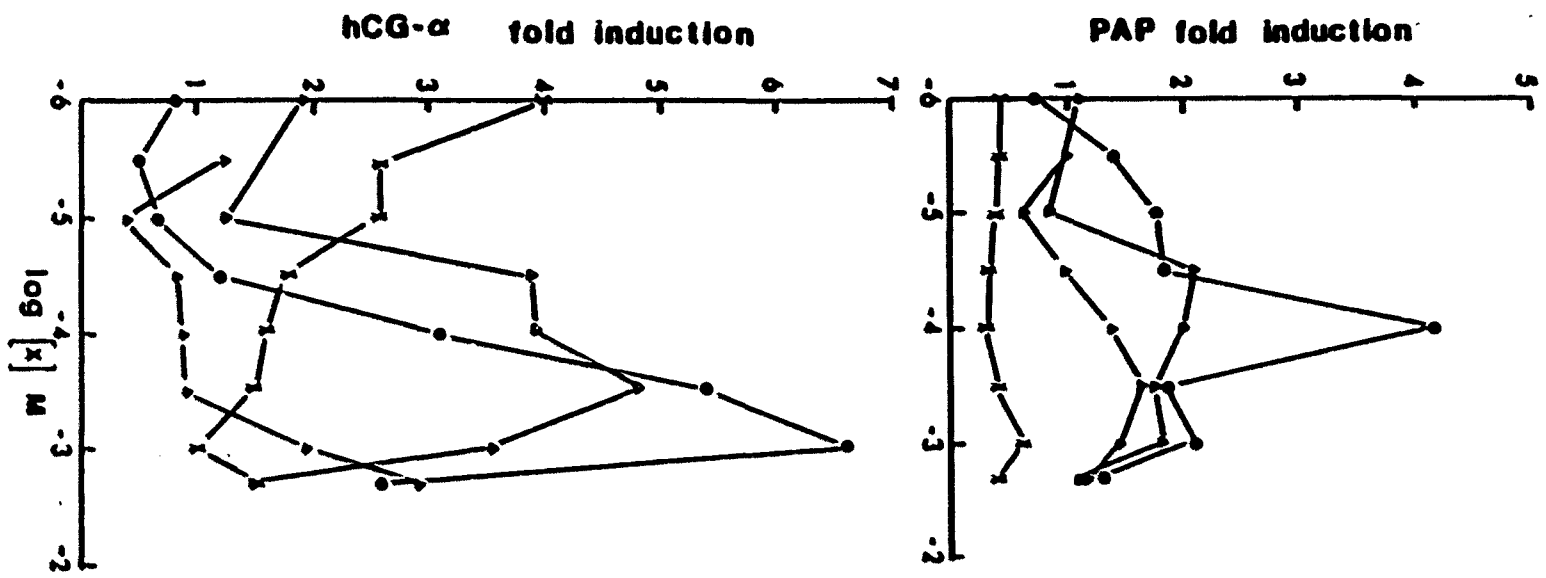
12 hours. Subsequent synthesis continued at a rate higher than that with butyrate or theophylline alone. Thus, the combination of butyrate and theophylline stimulated the production of hCG- α earlier than either compound alone, reducing the lag time by about 12 h. These data also suggest that theophylline and thymidine are interacting with butyrate to stimulate α production via different mechanisms.

Ectopic protein induction by thymidine, bromodeoxyuridine, fluorodeoxyuridine, and iododeoxyuridine

Many of the agents observed to induce ectopic proteins in HeLa cells are compounds which inhibit nucleotide metabolizing enzymes or are themselves modified deoxynucleosides (Park, 1981). As such, a number of these have proven to be useful chemotherapeutic agents (e.g. fluorodeoxyuridine, hydroxyurea, bromodeoxyuridine, cytosine arabinoside).

A study of the concentration effect of the deoxynucleoside analogs thymidine, bromodeoxyuridine, fluorodeoxyuridine, and iododeoxyuridine on the induction of hCG- α and PAP produced curves similar to those for butyrate (Fig 13). That is, the induction of hCG- α and PAP was dose-dependent, with maximum PAP production occurring at a lower concentration of inducer than maximum hCG- α induction. Concentrations which gave maximum induction of PAP and alpha subunit were 100 μ M and 1 mM respectively for IdUrd, 30-100 μ M and 300 μ M for BrdUrd, and 300 μ M and 2 mM for Thd. The results obtained with FdUrd reflect the

Figure 13. Induction of hCG- α and PAP by thymidine, iododeoxyuridine, fluorodeoxyuridine, and bromodeoxyuridine. [HeLa S-3 cells were grown in the presence of 1 μ M to 2 mM Thd, IdUrd, FdUrd, or BrdUrd according to the induction protocol in Materials and Methods. The results are plotted as the fold induction of control cultures which contained (A) 6.9 mU/mg protein for PAP activity and (B) 302 ng/mg protein for hCG- α . Symbols: Thd (Δ), IdUrd (\bullet), FdUrd (X), BrdUrd (Δ).]



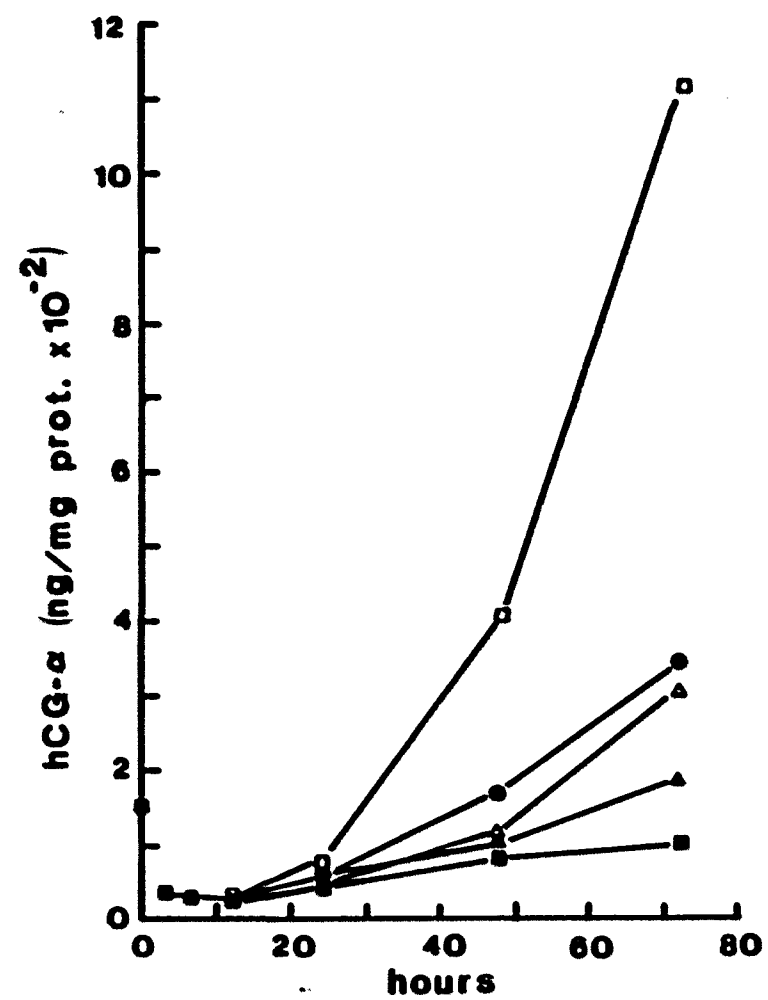
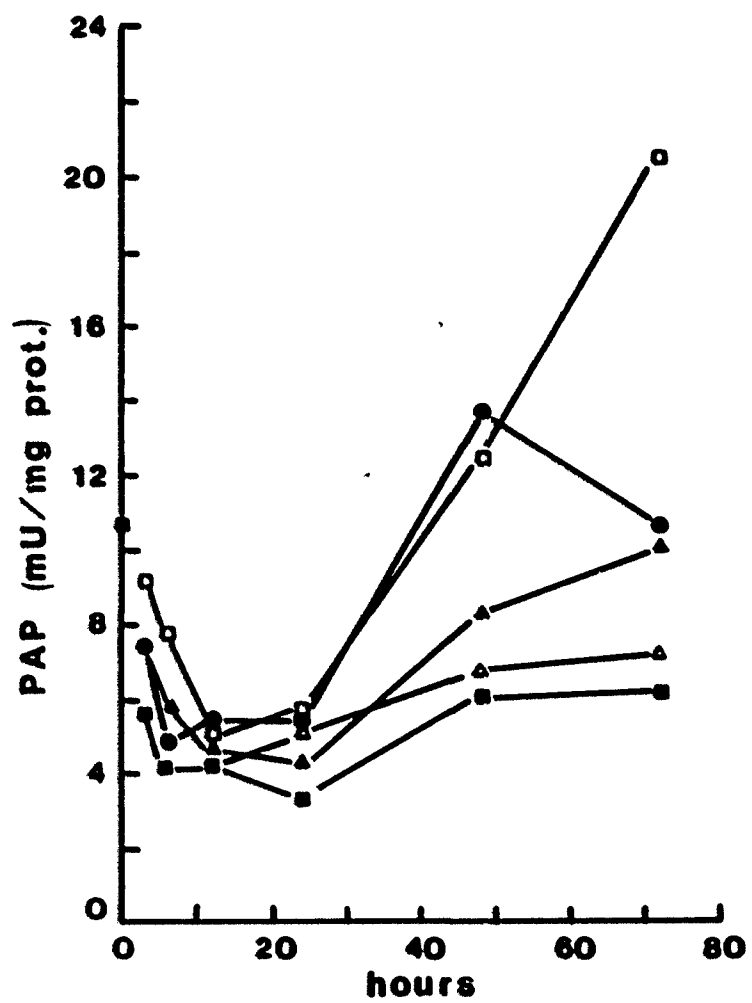
cytotoxicity of this compound as indicated by the cell necrosis and death in cultures containing at least 3 μ M. These results are a further indication that there are differences in the regulation of alpha and PAP gene expression.

The kinetics for protein induction in response to these deoxyuridine derivatives is illustrated in Fig 14. In general, it took longer for cells to respond to the deoxyuridine compounds than to butyrate. The carboxylic acid induced hCG- α and PAP between 12 and 24 hours after addition and supported higher protein levels than those realized with any of the deoxyuridine derivatives. Iododeoxyuridine induced hCG- α between 24 and 48 hours after addition while thymidine and bromodeoxyuridine induced hCG- α between 48 and 72 hours. The time course of induction of PAP was less clear. The increase in enzyme activity in response to IdUrd and butyrate were similar for at least 48 hours. The former showed a slight decrease in activity at 72 hours. Thymidine induced PAP between 24 and 48 hours and BrdUrd did not induce PAP in this experiment.

Inhibition of induction by deoxycytidine

In a recent report, Goz et al. (1980) reported that deoxycytidine inhibited the induction of PAP by IdUrd, butyrate, hydrocortisone, and choline chloride in HeLa cells. Along with dCyd, other nucleosides including thymidine, cytidine, and cytosine arabinoside inhibited the induction of alkaline phosphatase by IdUrd in a dose dependent manner. While these results may be explained by an expansion of thymidine pools

Figure 14. Time course of hCG- α and PAP induction by butyrate, IdUrd, Thd, and BrdUrd. [HeLa S-3 cells were grown to near confluency and refed with fresh MEMC or MEMC containing 3 mM butyrate, 100 μ M IdUrd, 100 μ M BrdUrd, or 1 mM Thd. Following these additions, cultures were harvested at 1, 5, 12, 24, 48, and 72 h and assayed for hCG- α and PAP as described in Materials and Methods. Symbols: Control (■), Btr (□), IdUrd (●), Thd (▲), BrdUrd (Δ).]



to dilute the incorporation of IdUrd into DNA, the correlation between IdUrd incorporation and alkaline phosphatase induction was non-linear. From these data, Goz et al. suggested that incorporation of IdUrd into DNA was not required for alkaline phosphatase induction and that cytidine and dCyd had another, undefined, locus for the inhibition of alkaline phosphatase induction. The effect of dCyd on α induction had not previously been examined. Consequently, it was of interest to determine whether dCyd had an effect on the induction of PAP and hCG- α by the various agents under investigation in the present study. Table IV shows that, in general, 2 mM dCyd inhibited the induction of both PAP and hCG- α . The exception may be the induction of hCG- α by butyrate or hydroxyurea, where no inhibition by dCyd was observed with butyrate alone. Furthermore, partial inhibition was observed with hydroxyurea alone or when butyrate was combined with another inducer. Comparison of the effect of dCyd on butyrate between the two experiments reflects the variability observed, with the data of Experiment 1 representative of the majority of cases. A t-test analysis of the effect of dCyd on the Btr induction of PAP and hCG- α gave a t value of 1.46 with $p \leq 0.084$ and a t value of 0.31 with $p > 0.25$, respectively. These data suggest that dCyd may be affecting a process common to the induction of PAP and hCG- α but that when butyrate is present, the induction of hormone subunit is less sensitive than is that of PAP. To study this further, experiments were concentrated on the effect of dCyd on the butyrate induction of hCG- α and PAP.

Table IV
Effect of deoxycytidine on the induction of hCG- α and PAP^a

Sample	PAP	hCG- α
Experiment I	mU/mg protein	ng/mg protein
Control	8.6 \pm 8.3 (24)	226 \pm 122 (22)
dCyd 2 mM	7.8 \pm 6.3 (15)	220 \pm 131 (14)
Btr 3 mM	24 \pm 24 (24)	1460 \pm 1010 (22)
Btr + dCyd	14 \pm 11 (16)	1300 \pm 2080 (16)
Experiment II	fold induction	fold induction
Control	1.0	1.0
dCyd 2 mM	0.5	1.2
Btr 3 mM	3.7	9.0
Btr + dCyd	2.7	17.8
BrdUrd 0.1 mM	1.6	6.7
BrdUrd + dCyd	0.8	1.9
IdUrd 0.1 mM	2.1	5.4
IdUrd + dCyd	1.0	1.6
Btr + IdUrd	3.7	19.2
Btr + IdUrd + dCyd	2.1	25.3
Thd 0.3 mM	1.7	10.9
Thd + dCyd	0.8	1.8
Btr + Thd	3.5	26.1
Btr + Thd + dCyd	1.8	16.2
Theophylline	0.8	9.6
Btr + theophylline	2.7	58.7
Btr + theophylline + dCyd	1.4	35.0
hydroxyurea	1.2	8.4
hydroxyurea + dCyd	0.8	5.1

^aHeLa S-3 cells in 25 cm² flasks were grown as described in Materials and Methods. In Experiment I, the number of replicates is given in parenthesis. Control values were 502 ng/mg protein for hCG- α and 12.8 mU/mg protein for PAP in Experiment II.

Fig 15 shows the dose-response for dCyd on the induction of PAP and hCG- α in the presence and absence of butyrate. While the induction of both PAP and hCG- α was inhibited by dCyd, PAP was more sensitive than hCG- α . That is, the concentration of dCyd (2 mM) which inhibited PAP induction by 50% was half the concentration (4 mM) which inhibited the hCG- α induction by 50%.

The effect of dCyd on the time course for butyrate induction of PAP and hCG- α is shown in Fig 16. The alpha subunit was induced 12 to 24 hours after the addition of butyrate in both butyrate and Btr/dCyd treated cells; production rates remained similar for 48 hours but decreased slightly between 48 and 72 hours in Btr/dCyd cultures. In contrast, PAP was induced 24 to 48 hours after the addition of butyrate and this increase was abolished completely when the cultures were supplemented with 2 mM dCyd. The inhibition of PAP and hCG- α did not result from a general cytotoxic effect on the cells (Table V). Deoxycytidine inhibited DNA synthesis completely, RNA synthesis only slightly, and had little or no effect on protein synthesis. Based on work by others in this laboratory, the degree of RNA synthesis inhibition was not considered to be sufficient to account for the inhibition of PAP induction.

There are several mechanisms by which dCyd might alter the cellular response to butyrate. The nucleoside might somehow interfere with the uptake or metabolism of the fatty acid and thereby inhibit protein induction. Consequently, the incorporation of [^{14}C] butyrate in the

Figure 15. Effect of deoxycytidine on the butyrate induction of hCG- α and PAP. [HeLa 2.2 cells in 25 cm² flasks were grown in dCyd alone (1 to 4 mM) or in combination with 3 mM butyrate. Cultures were harvested 72 h later and assayed for PAP and hCG- α as described in Materials and Methods. Control values were (A) 0.56 mU/mg protein for PAP and (B) 328 ng/mg protein for hCG- α . Butyrate induced hCG- α about 4 fold and PAP about 19.4 fold. Cultures grown in the absence or presence on butyrate are represented by the closed (● ▲) or open (○ △) symbols, respectively.]

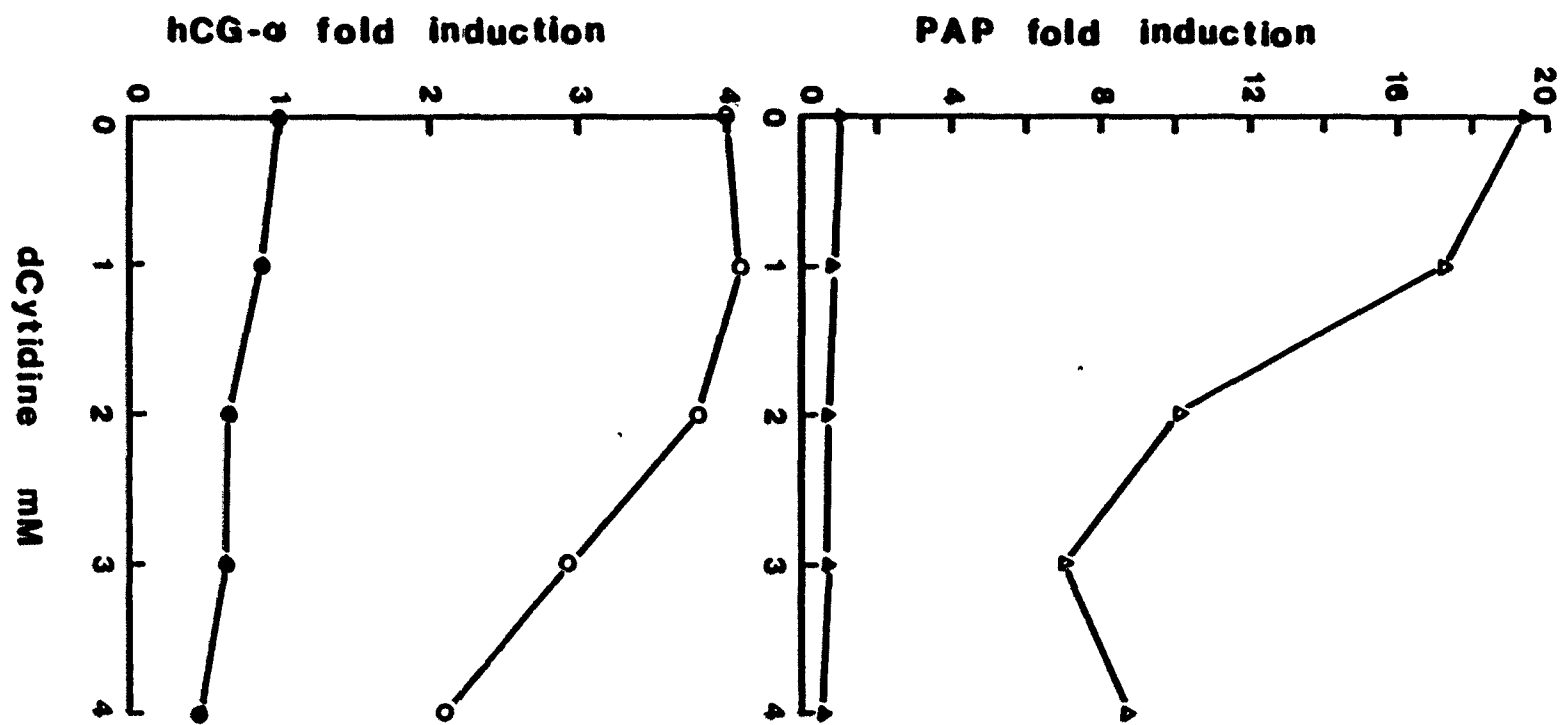


Figure 16. Effect of dCyd on the time course of induction of PAP and hCG- α by butyrate. [HeLa S-3 cells were grown in MEMC alone (Control) or MEMC containing 3 mM butyrate and/or 2 mM dCyd. Cultures were harvested at 6, 15, 24, 48, and 72 h and assayed for (A) PAP and (B) hCG- α . Symbols: Control (●), dCyd (■), Btr (○), Btr + dCyd (□).]

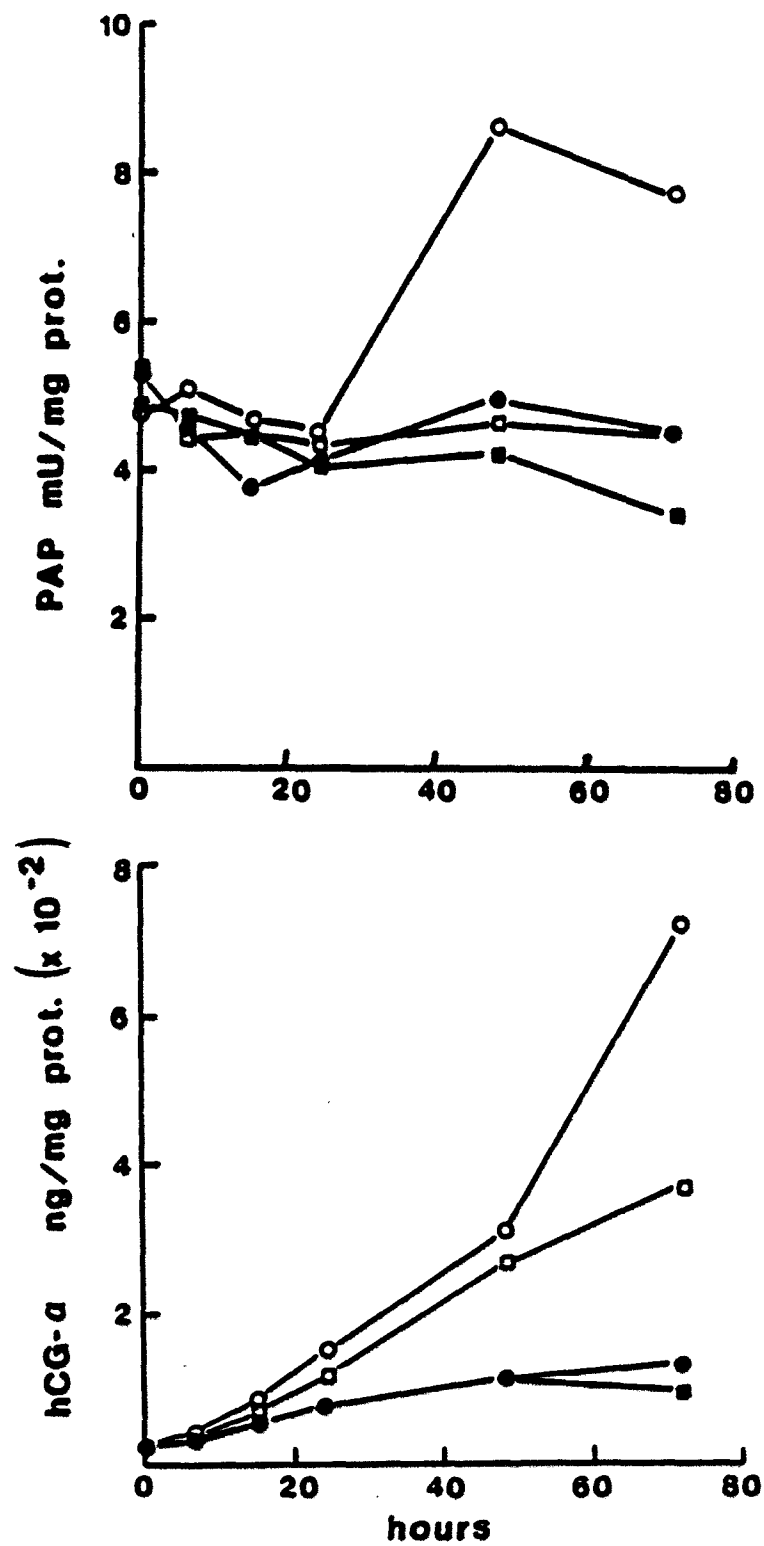


Table V

Effect of deoxycytidine on DNA, RNA, and protein synthesis^a

Sample		Macromolecular Synthesis		
		DNA	RNA	Protein
Control		100	100	100
+ dCyd (mM)	0.1	10	72	81
	0.5	4	68	68
	1	4	62	92
	2	3	63	99
Butyrate		48(100)	129(100)	171(100)
+ dCyd (mM)	0.1	6(13)	104(81)	129(74)
	0.5	6(13)	88(68)	122(70)
	1	5(11)	78(61)	128(73)
	2	4(9)	63(49)	128(73)

^aHeLa S-3 cells in 24 well culture flasks were treated with dCyd alone or in combination with butyrate (3 mM) for 3 days. Individual wells were labeled with [³H] thymidine or [³H] uridine and [¹⁴C] amino acid mixture for 4 h. The samples were harvested and assayed as described in Materials and Methods. Control values were 1.66×10^5 cpm/mg protein for [³H] thymidine incorporation, 3.15×10^4 cpm/mg protein for [³H] uridine incorporation, and 1.92×10^4 cpm/mg protein for [¹⁴C] amino acid incorporation.

absence and presence of dCyd was investigated. Table VI shows that dCyd alone did not inhibit the cellular incorporation of butyrate at 9 or 30 hours after the addition of dCyd.

To determine if an inhibitor of PAP was present in dCyd treated cells, sonic extracts of cells cultured for 72 h with 3 mM butyrate, 2 mM dCyd, or Btr/dCyd in combination were mixed together in different ratios and assayed for alkaline phosphatase activity. The observed activity was compared with the values expected to be present based on the ratio of each sonicate (Table VII). The nearly identical values for observed and expected activity suggested that a freely diffusible inhibitor of alkaline phosphatase activity was not present in dCyd treated cells. Deoxycytidine also did not have a direct effect on commercially available PAP or PAP partially purified from Hela or human placenta (data not shown).

The inhibition of PAP induction by dCyd can be prevented by preincubating the cells with butyrate (Fig 17). Addition of 2 mM dCyd within 6 h of the addition of butyrate inhibited the induction of PAP activity. However, if the cells were exposed to butyrate for 11 h before the addition of dCyd, an inhibition of PAP activity was not observed. Thus, dCyd interferes with an early event of butyrate induction. This event occurs within 6 to 11 h after the addition of butyrate and it is resistant to the subsequent addition of dCyd. Preincubation with butyrate had little effect on the induction of hCG- α .

Table VI

Effect of dCyd on the incorporation of [^{14}C] butyrate into HeLa cells^a

Sample	[^{14}C] butyrate incorporation	
	cpm/ 10^5 cells	
	9 h	30 h
Control	4500	3100
dCyd 30 μM	4900	3300
100 μM	5300	3100
2000 μM	4900	3700

^aHeLa S-3 cells were grown in 24-well titer plates in the presence of 30 μM , 100 μM , and 2 mM dCyd. After either 7 or 28 h, the cells were labelled with 1 μCi of [^{14}C] butyrate for 4 h. The cells were harvested, collected on Millipore filters, and counted in a liquid scintillation fluor.

Table VII
PAP activity in mixtures of cell sonicates^a

Ratio of sonicates	PAP mU/ml	
	Observed	Expected
Btr/dCyd : Btr		
1 : 0	9.33	9.33
3 : 1	11.42	11.40
1 : 1	13.21	13.48
1 : 3	13.73	13.56
0 : 1	17.63	17.63
dCyd : Btr		
1 : 0	13.21	13.21
3 : 1	13.91	14.31
1 : 1	15.55	15.42
1 : 3	17.02	16.52
0 : 1	17.63	17.63

^aSonic extracts of cells grown in 3 mM butyrate were mixed with those of cells grown in 2 mM dCyd or butyrate plus dCyd. After 30 min on ice, the samples were assayed for alkaline phosphatase activity. The observed PAP was compared with the activity expected to be present based on the ratio of the sonicates.

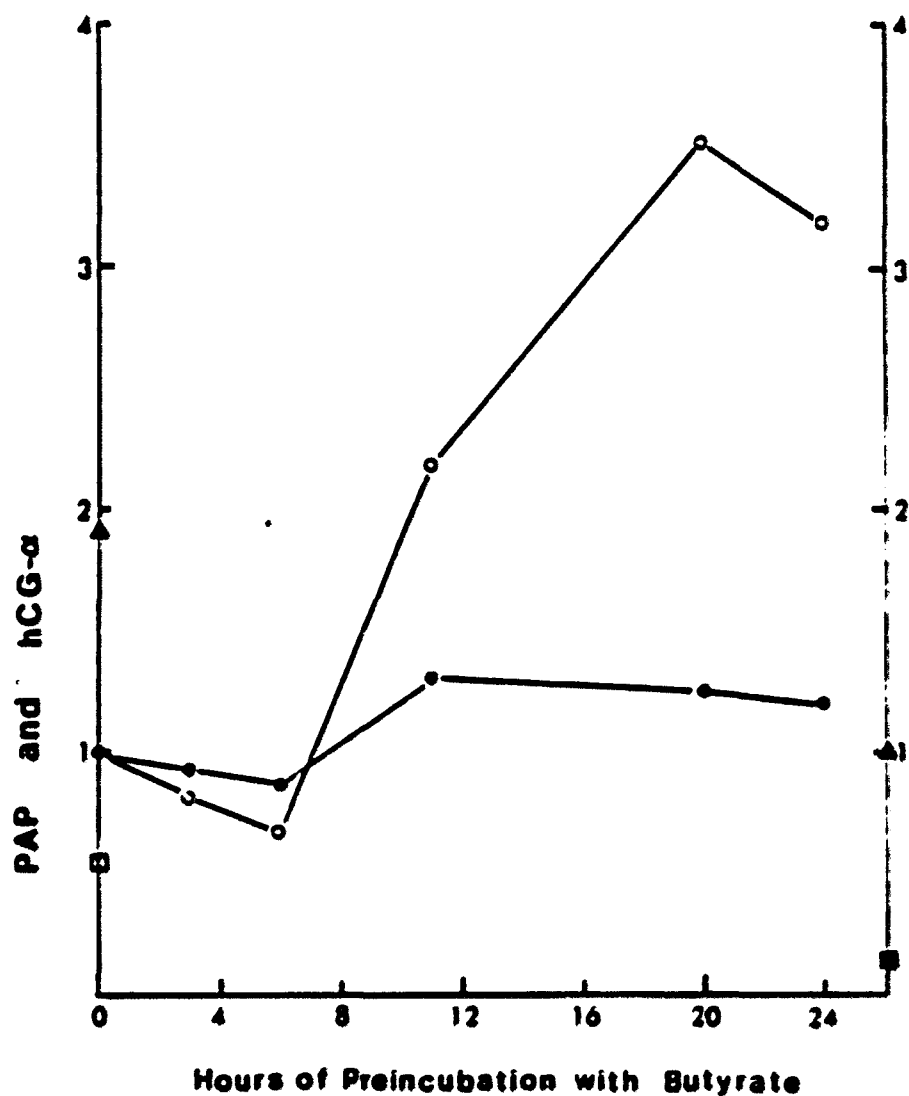


Figure 17. Preincubation with butyrate before the addition of dCyd. [HeLa S-3 cells were fed with 3 mM butyrate at time zero. Deoxycytidine (2 mM) was added subsequent to butyrate at 0, 3, 6, 11, 20, and 24 h. The cultures were harvested after 72 h and assayed for PAP activity and hCG- α as described in Materials and Methods. The results are expressed relative to the levels obtained in cultures receiving But plus dCyd at time zero; i.e., no preincubation with But. Control values were 99 ng/mg protein for hCG- α and 2.1 mU/mg protein for PAP. Symbols: PAP (○), hCG- α (●), Control (□ or ■), and But (△ or ▲) levels for PAP (□, △) and hCG- α (■, ▲) respectively.]

Butyrate mediates numerous changes in cellular membranes, such as the induction of sialyltransferase I and cholera toxin receptors (Fishman et al., 1974; Fishman and Atikkan, 1979). Also, Wharton and Goz (1978) and Hung and Melnykovych (1976) reported that choline and lysophosphatidylcholine respectively stimulated PAP activity in HeLa cells. Since the nucleotide derivative CDP-choline is an intermediate of phosphatidylcholine synthesis, the effect of butyrate and dCyd on the incorporation of [^{14}C] choline was examined. During the initial 36 hours of exposure, butyrate inhibited the incorporation of choline but dCyd did not reverse this inhibition (Fig 18).

Other deoxynucleosides in addition to dCyd inhibited the butyrate induction of PAP activity (Table VIII). Deoxyadenosine and deoxyguanosine inhibited hCG- α and PAP induction at 2 mM but not at 100 μM . Deoxycytidine inhibited the PAP induction at 2 mM but not at 100 μM and did not affect hCG- α induction at either concentration. Thymidine did not affect the induction of PAP and was slightly synergistic with butyrate for the induction of hCG- α . It appears that, in general, purines are more effective inhibitors of the butyrate induction of PAP activity and hCG- α than pyrimidines.

Inhibition of DNA synthesis

Since protein induction has been postulated to be correlated with the inhibition of DNA synthesis (Ghosh et al., 1977), it was of interest to determine the effect of the compounds used in this study on DNA synthesis. It was of particular interest to see whether the antagonism

Figure 18. Effect of dCyd on the incorporation of [14 C] choline
[HeLa S-3 cells were grown in 24-well cluster plates in the presence of dCyd alone or in combination with 3 mM Btr. Cells were labelled for 4 h with 1 μ Ci of [14 C] choline at 8, 22, and 34 h after Btr and/or dCyd addition, harvested, collected on Millipore filters, and counted in a liquid scintillation fluor. Symbols: Control (∇), 100 μ M dCyd (\bullet), 500 μ M dCyd (\blacksquare), 2 mM dCyd (\blacktriangle), 4 mM dCyd (\times), Btr (∇), Btr + 100 μ M dCyd (\circ), Btr + 500 μ M dCyd (\square), Btr + 2 mM dCyd (Δ).]

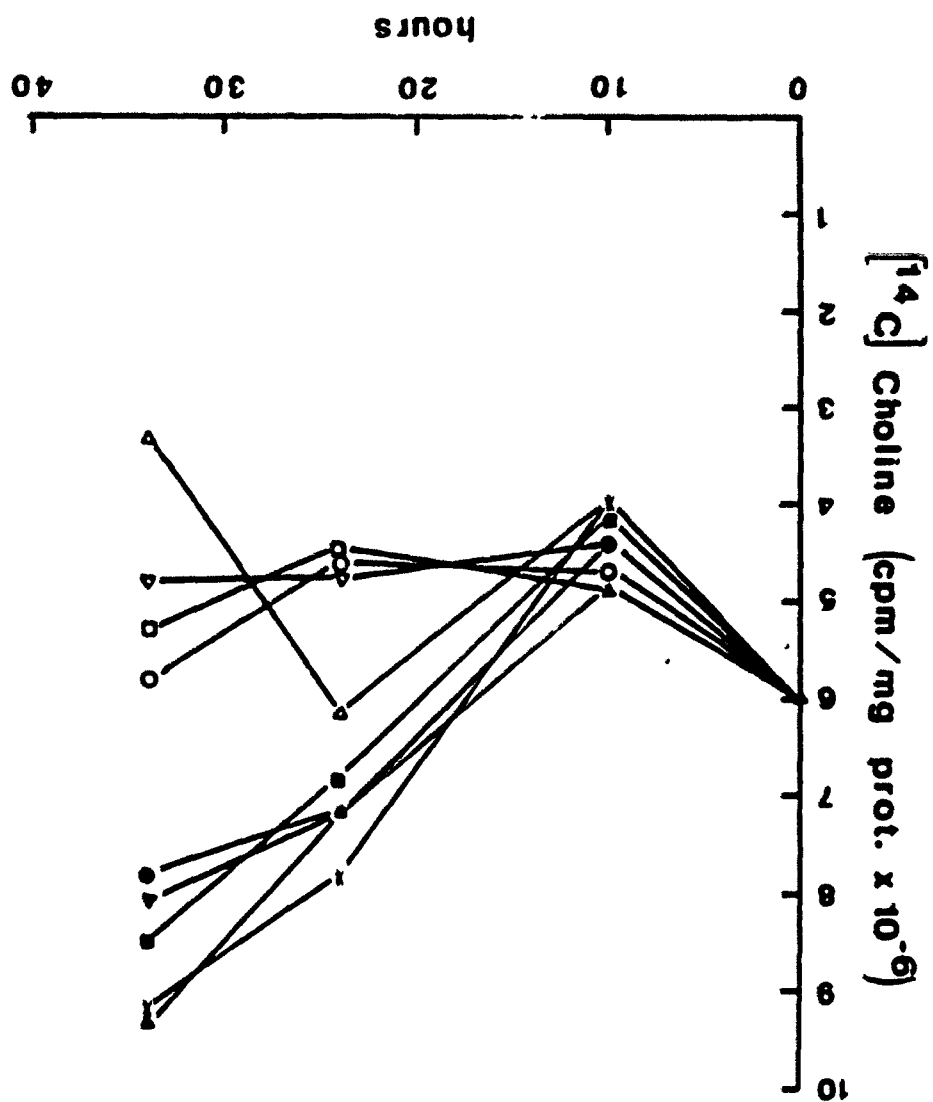


Table VIII

Effect of deoxynucleosides on the butyrate induction of hCG- α and PAP^a

Sample	Fold induction	
	hCG- α	PAP
Control	1.0	1.0
dCyd 0.1 mM	1.0	0.9
2 mM	0.5	0.9
Thd 0.1 mM	0.9	0.4
2 mM	1.7	1.7
dAdo 0.1 mM	0.5	0.6
2 mM	0.6	1.0
dGuo 0.1 mM	1.0	0.9
2 mM	0.3	0.8
Bcr 3 mM	3.0	29.4
Bcr + dCyd 0.1 mM	2.8	27.3
Bcr + dCyd 2 mM	4.3	15.1
Bcr + Thd 0.1 mM	4.0	18.8
Bcr + Thd 2 mM	5.8	33.0
Bcr + dAdo 0.1 mM	2.5	33.4
Bcr + dAdo 2 mM	1.9	13.0
Bcr + dGuo 0.1 mM	3.1	31.8
Bcr + dGuo 2 mM	0.8	3.4

^aHeLa 2.2 cells in 25 cm² flasks were treated with deoxynucleosides alone or in combination with Bcr for 3 days. Cells were harvested and assayed for hCG- α as described in Materials and Methods. PAP activity was measured as described in Table III. The A₄₀₀ was recorded manually at 1 to 2 min intervals. The non-enzymatic hydrolysis of p-nitrophenylphosphate was determined using 100 μ l of water instead of cell sonicate. A mM extinction coefficient of 13 was used for p-nitrophenol (see Table III). Control values were 1.15 μ g/mg protein for hCG- α and 0.61 mU/mg protein for PAP.

between dCyd and other inducers could be explained, in part, by a reversal of the DNA synthesis inhibition. Table IX shows that most of the compounds inhibited the incorporation of radiolabeled precursors into TCA precipitable material from 40-60% compared to control levels at concentrations which affect ectopic protein expression. Those compounds which did not inhibit DNA synthesis as strongly included 1 mM theophylline, 2 mM dCyd, Thd (1-2 mM), 1 mM IdUrd, 3 mM hydroxyurea, and possibly the combination of butyrate and theophylline. The difference in the data between the two butyrate plus theophylline combinations may reflect the difference in how the experiments were done (incubation with effectors for 24 h vs. 3 days prior to isotope addition; different radiolabeled precursors). Combination of effectors enhanced the inhibition of DNA synthesis. The addition of dCyd did not reverse the inhibition of DNA synthesis. By comparing the inhibition of DNA synthesis with the induction of α and PAP (Figures 10, 11, and 13; Table IV), there does not appear to be a correlation between the degree of induction and the inhibition of DNA synthesis suggesting that these two events are independent of one another.

Levels of cAMP

While the cAMP (Fig 11) and methylxanthine data (Table III) suggest that cAMP is not involved in the production of hCG- α and PAP, measurement of intracellular cAMP levels under a variety of culture conditions should help to clarify the situation. The intracellular level of cAMP was measured in cells grown under various induction

Table IX
Effect of inducers on DNA synthesis^a

Sample	Percent of control	
	[³ H] Thd	[³ H] dAdo
Experiment I		
Control	100	100
Bcr	49	65
dAdo 2 mM	50	
Bcr + dAdo	43	
dGuo 2 mM	21	
Bcr + dGuo	15	
theophylline 1 mM	89	
Bcr + theophylline	74	
dCyd		78
Bcr + dCyd		32
Thd 1 mM		86
2 mM		68
Bcr + Thd 2 mM		27
IdUrd 1 mM		84
2 mM		49
BrdUrd 1 mM		47
2 mM		52
Experiment II		
Control		100
HU 3 mM		66
dCyd 2 mM		82
Bcr + dCyd		52
HU + dCyd		54
Bcr + HU		44
Bcr + HU + dCyd		28
theophylline		105
Bcr + theophylline		50

^aIn Experiment I, HeLa 2.2 cells in 24 well culture dishes were grown under the experimental conditions for 24 h and labelled with 1 μ Ci [³H] Thd or 1 Ci [³H] dAdo for 4 h. The medium was removed by aspiration and 0.5 ml of 0.3 N KOH was added to each well. After 20 to 46 h at room temperature, the KOH extract was neutralized with 150 μ L of 1 N HCl. A 50 μ L aliquot was removed for a Lowry protein determination and the rest of the sample was precipitated with 1 ml of ice cold 10% TCA for 30 min, collected on Millipore nitrocellulose filter, dried, and counted in liquid scintillation fluor. Control values were 3900 cpm/mg protein for [³H] Thd incorporation and 912 cpm/mg protein for [³H] dAdo incorporation. In Experiment II, cells were harvested and assayed after 3 days growth as described in Materials and Methods. Control value was 2.49×10^5 cpm/mg protein.

conditions (Table X). Although the level of cAMP differs from one condition to another, the magnitude of the changes does not seem to be sufficient to account for the induction of proteins or the inhibition of PAP induction by dCyd and theophylline. Theophylline increased cAMP to similar levels by itself and in combination with butyrate, but production of hCG- α was 11 fold greater in combination with butyrate (Fig 12). The combinations of Btr/dCyd and Btr/Thd also had comparable levels of cAMP. However, Btr/Thd cultures had higher levels of α than Btr/dCyd and the butyrate induction of PAP was inhibited by dCyd but not Thd. Coupled with the cAMP and methylxanthine addition experiments, these data support the view that the synergism between theophylline and butyrate for the induction of hCG- α involves a mechanism other than cAMP production.

Deoxynucleotide levels in control and butyrate cells

Most of the effectors of ectopic protein synthesis used in this study were nucleoside compounds. In other systems, the addition of excess deoxynucleosides profoundly altered endogenous pool sizes (Snyder, 1984; Cohen et al., 1983). To determine if butyrate also affected the concentration of deoxynucleotides, acid soluble extracts of control and butyrate treated cells were assayed for deoxynucleoside triphosphates as described in Materials and Methods. Control cells contained about 36.4 pmoles dATP/mg protein and 8.0 pmoles dTTP/mg protein. Butyrate-treated cells contained about 14.6 pmoles dATP/mg protein and 6.2 pmoles dTTP/mg protein. The levels of dCTP and dGTP

Table X

Effect of protein inducers on the levels of cAMP^a

Sample	cAMP (pmoles/mg protein)
Control	2.31
dCyd	2.40
Btr	2.40
Btr + dCyd	3.16
theophylline	3.87
Btr + theophylline	4.14
Thd	2.71
Btr + Thd	3.13

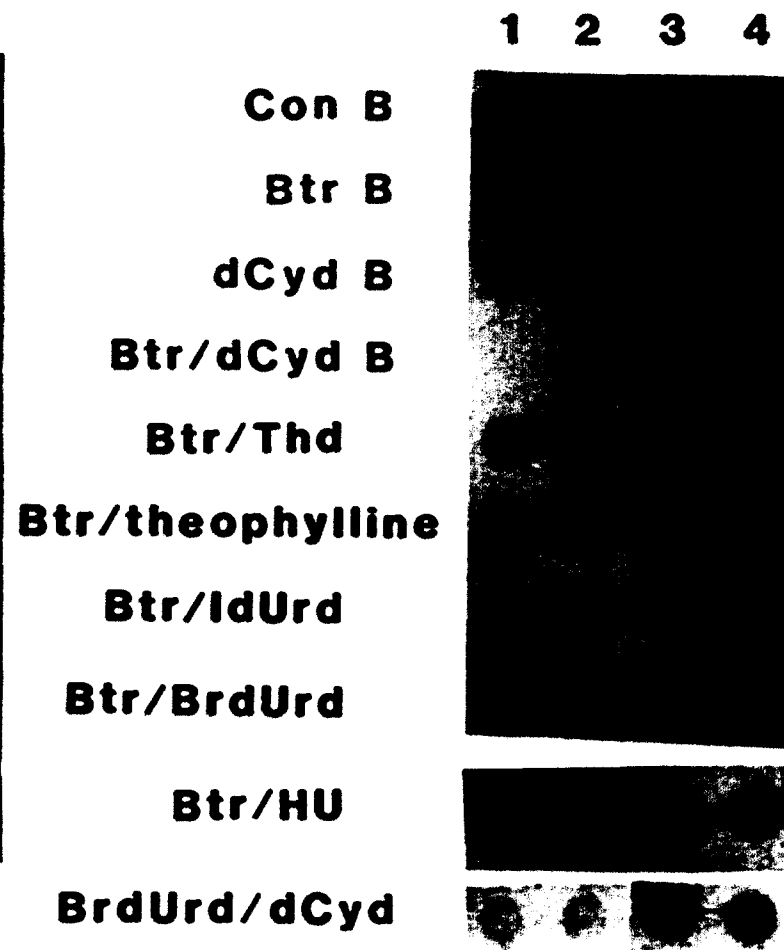
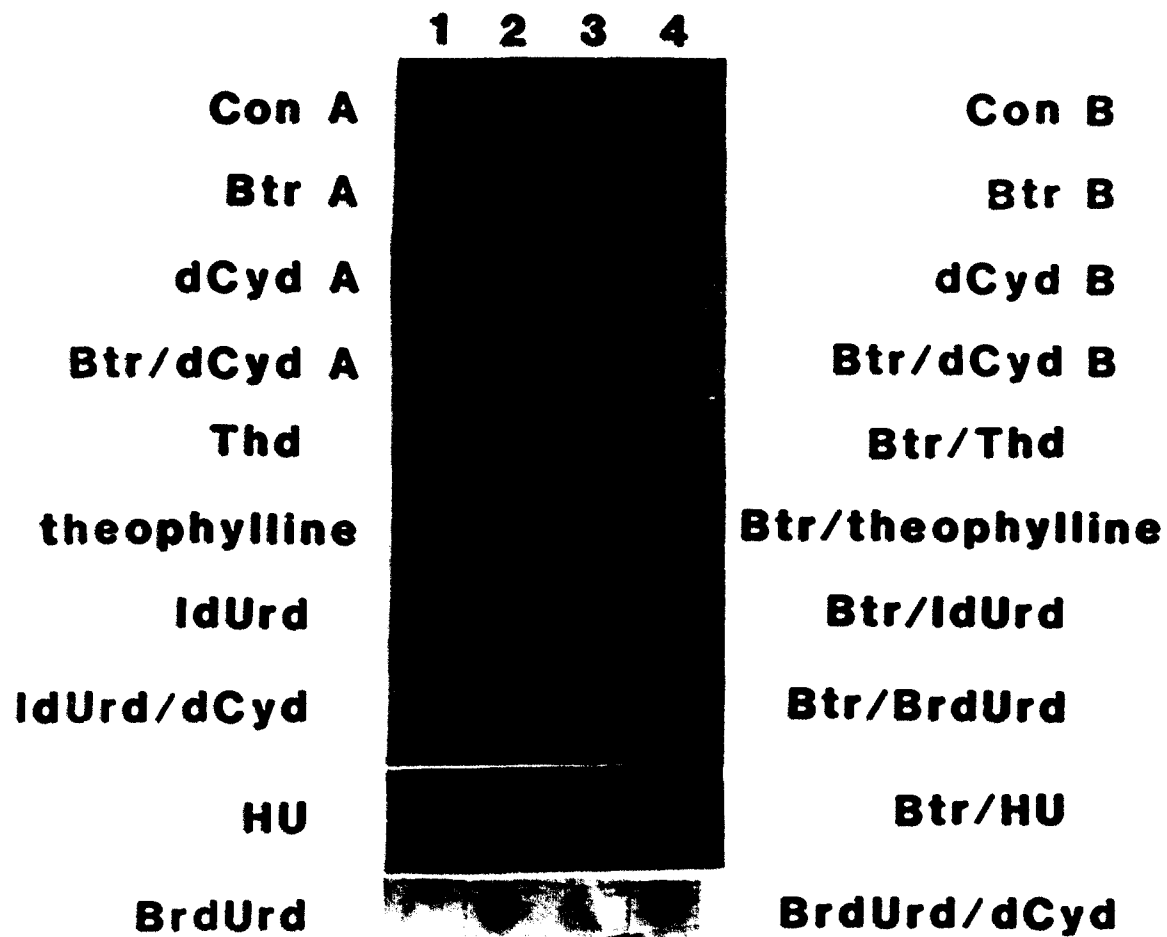
^aHeLa 2.2 cells in 75 cm² flasks were treated with 2 mM dCyd, 1 mM theophylline, 2 mM Thd, alone or in combination with 3 mM Btr for 3 days. The cells were harvested and assayed for cAMP as described in Materials and Methods.

were not detectable in either control or butyrate treated cells. This decrease in dATP and dTTP may reflect the inhibition of cell growth mediated by butyrate, possibly resulting from or leading to the inhibition of DNA synthesis, but it is unclear whether the levels of dATP, dTTP, or other deoxynucleoside triphosphate is significant to the expression of ectopic proteins.

Detection of hCG- α transcripts in cytoplasmic extracts

Although there are many possible sites to regulate gene expression in eukaryotes, the most frequent level of control appears to be transcriptional (Darnell, 1982). To assess the possible transcriptional regulation of α , the effect of various inducers on the levels of α -mRNA was examined using the cytoplasmic dot hybridization technique described by White and Bancroft (1982). Messenger RNA in cytoplasmic extracts was immobilized on nitrocellulose and hybridized to ^{32}P -labeled α -cDNA probe. The level of hCG- α transcripts in each sample was reflected by the density of the autoradiograph emulsion (Fig 19). Although the densities are very similar for many of the samples, there may be a slight increase above the control samples in the extracts of cells grown in butyrate, theophylline, and IdUrd. A significant difference in the densities was present in those cells with greatly enhanced rates of hCG- α protein production (Btr/theophylline; Btr/BrdUrd; Btr/IdUrd; Btr/HU). Scanning the autoradiograph with a laser densitometer and comparing the relative densities of each sample with the corresponding fold increase in hCG- α protein production supports these observations

Figure 19. Detection of hCG- α mRNA by cytoplasmic dot hybridization. [HeLa S-3 cells were grown in the presence of 3 mM Btr, 2 mM dCyd, Btr + dCyd, 1 mM IdUrd, IdUrd + dCyd, Btr + IdUrd, 300 μ M BrdUrd, BrdUrd + dCyd, Btr + BrdUrd, 2 mM Thd, Btr + Thd, 1 mM theophylline, Btr + theophylline, 3 mM HU, and Btr + HU according to the induction protocol described in Materials and Methods. The filters were probed with nick translated α -cDNA plasmid (1.4×10^8 cpm / μ g). Samples were diluted 1/50 in column 1, 1/15 in column 2, 1/7.5 in column 3, and 1/5 in column 4. A and B refer to two separate experiments.]



(Table XI). Since the densities of the autoradiograph emulsions depend on the length of exposure as well as the amount of bound radiolabeled material, differences in the scanning data between the darkest spots and the control samples may be minimum values due to over-exposure.

However, a plot of the data in Table XI (omitting the Btr + theophylline values) generates a curve with a linear regression correlation coefficient of 0.92 (Fig 20). Hence, these data suggest that, in general, increases in α subunit correspond to increases in α -mRNA.

Table XI

Comparison of dot blot intensities with induction of hCG- α protein^a

Sample	Fold Induction	
	α -mRNA relative film intensity	normalized hCG- α protein
Control A and B	1.00	1.00
Btr A	1.43	6.5
B	2.22	8.6
dCyd A	0.23	0.59
B	0.33	0.93
Btr + dCyd A	1.08	5.43
B	-	-
BrdUrd	0.78	1.52
BrdUrd + dCyd	1.47	1.54
Btr + BrdUrd	5.99	16.3
IdUrd	2.03	3.88
IdUrd + dCyd	1.92	2.32
Btr + IdUrd	8.80	18.90
Thd	0.21	2.15
Btr + Thd	3.28	11.49
Theophylline	0.86	2.96
Btr + Theophylline	7.44	100.69
Hydroxyures	2.93	2.41
Btr + HU	6.38	14.60

^aThe dot blot autoradiograph (Fig 19) was scanned with a Zeineh soft laser scanning densitometer (LKB Instr) at two gain settings (2 and 9). The relative density of each spot was corrected for the dilution of the original sample and normalized to the amount of total cellular protein. Since these values varied from the lowest to the highest dilution, the value of a sample at a particular dilution (1/50, 1/15, 1/7.5, or 1/5) was divided by the average of the two control (A and B) values at that dilution. The numbers generated by this method for a specific sample were averaged and are listed above. No values are given for Btr + dCyd B because the aliquot used for the protein determination was lost. The amount of hCG- α in the medium was assayed by RIA as described in the Materials and Methods, normalized to the amount of total cellular protein, and divided by the average of the Control level.

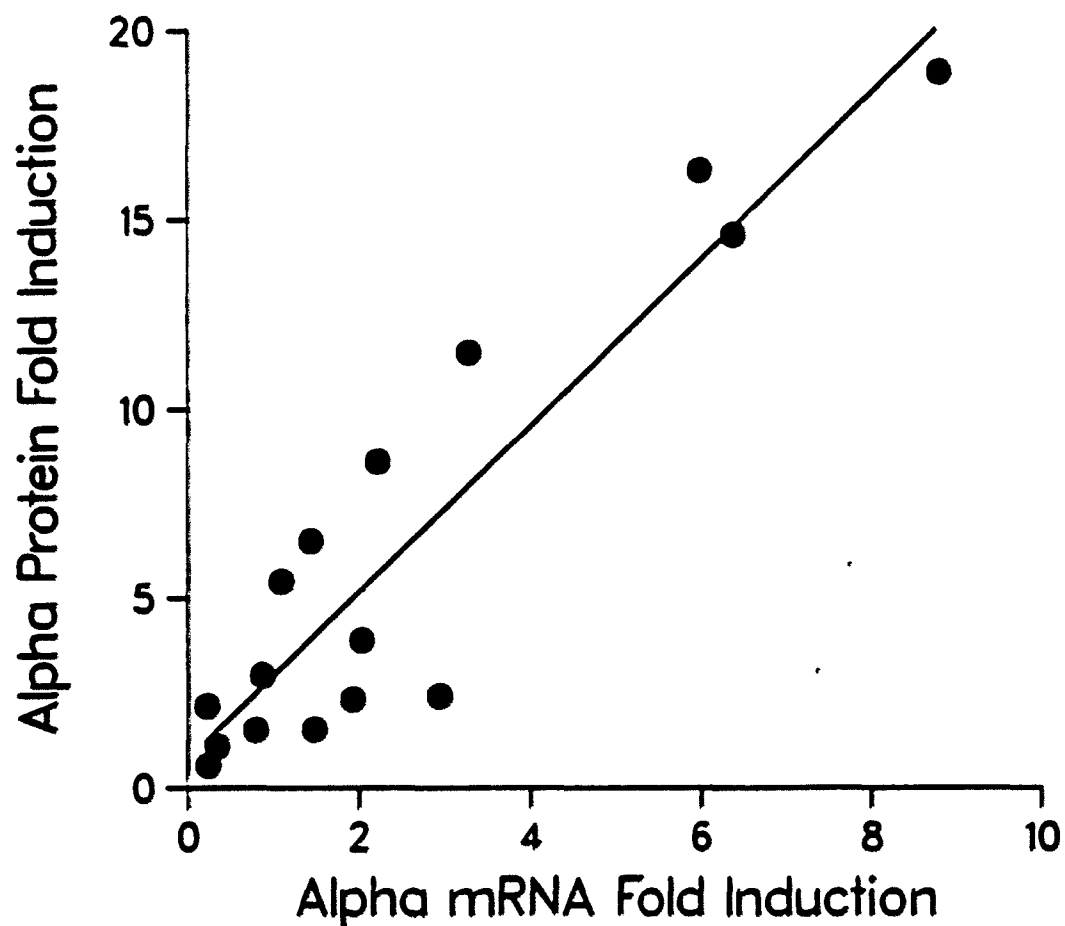


Figure 20. Comparison of alpha protein and mRNA induction. [Data of Table XI is presented graphically. Btr + theophylline sample was not plotted. The line was drawn by linear regression, with intercept, 0.87; slope, 2.2; correlation coefficient, 0.92.]

DISCUSSION

Variation in the constitutive production of hCG- α and PAP activity among HeLa cell lines as observed in this study (Table I) confirms observations made by others (Chou et al., 1977; Lieblisch et al., 1977; Lieblisch et al., 1976). However, the reason for this variation is unknown. From the initiation of transcription to the secretion of hCG- α into the medium or the insertion of PAP into the plasma membrane, there exists a number of possible sites of regulation. The similarity in size and apparent carbohydrate composition of the α -subunits synthesized by HeLa 2.1 (a low producer) and HeLa S-3 (a high producer) suggest that differences in their level of subunit production is not due to differences in the glycosylation mechanism (Fig 4-Fig 7). This is in contrast to work by Cox (1981c), who observed that the majority of desialylated HeLa- α from 2.1 medium did not bind to a column of Ricin-agarose, suggesting that the protein was deficient in terminal carbohydrates and different from the hCG- α produced by HeLa 2.2. The basis for this is unknown, but may reflect differences in the sample used for chromatography. Whereas Cox chromatographed dialyzed but unfractionated medium, the material used in this study for Ricin chromatography was partially purified and bound to a column of ConA Sepharose. These purification and chromatographic steps may have selected for material which would bind to Ricin. If such a partitioning occurred, samples examined in the previous study may have been more resistant to desialylation, possibly due to differences in the

carbohydrate composition (e.g., fucose residues in addition to or in lieu of sialic acid). It has also been observed that continuous culture of 2.1 cells leads to an increase in hCG- α production (unpublished observations). Thus, the difference may reflect the collection of medium from cells at different lengths of continuous culture. That is, hCG- α may be deficient in terminal carbohydrates when production is low but complete in terminal carbohydrates as production increases. A correlation between glycosylation and the production of hCG- α would be consistent with the previous report (Cox, 1981c) and work by Yoshimoto et al. (1979).

Induction of hCG- α and PAP by butyrate varied from one HeLa cell line to another (Fig 1) and the degree of induction did not correlate with the constitutive production rate (Table 1). The difference in the fold induction among the cell lines probably does not result from a variation in their uptake and/or metabolism of butyrate. Uptake of [14 C] butyrate was slower in HeLa 2.1 cells than in the other HeLa cell lines examined (data not presented), although the fold induction of hCG- α was comparable to HeLa 2.2. This indicates that even though a lower amount of butyrate is incorporated by 2.1 cells compared to 2.2 cells, it is sufficiently metabolized to induce hCG- α to comparable levels in the two cell lines. These widely different rates of constitutive production and response to butyrate among the HeLa cell lines suggests that they may be useful in identifying factors controlling the expression of hCG- α and PAP.

In this report, the production of hCG- α and PAP activity in HeLa cells was enhanced by butyrate, bromodeoxyuridine, iododeoxyuridine, and thymidine. With each of these compounds, a lower concentration of inducer was required for maximum PAP induction than for maximum hCG- α induction (Figures 10 and 13). The mechanism of induction by these compounds is unknown. The various effects of butyrate on cellular metabolism and possible effects on gene regulation were reviewed in the Introduction. With respect to the deoxynucleosides, some investigators have suggested that BrdUrd and IdUrd must be incorporated into the DNA in order to alter gene activity (Rutter et al., 1973; Bulmer et al., 1976; Bick, 1977), while others have disputed such a mechanism (Chou and Robinson, 1977a; Davidson and Kaufman, 1977; Goz et al., 1980). Since phosphorylation appears to be required for IdUrd action (Goz, 1974), deoxynucleotides, rather than the nucleosides, are most likely the active agents. The use, then, of cells deficient in thymidine kinase or other nucleoside metabolizing enzymes may be useful in determining the mechanism of action of these compounds.

The longer period of incubation with BrdUrd, IdUrd, and Thd than with butyrate needed to induce hCG- α and PAP implies that butyrate is more proficient than these deoxynucleosides (Fig 14). This may indicate that butyrate acts more directly on the expression of these proteins than the deoxynucleosides or that butyrate acts at multiple sites. The cytoplasmic dot blot data (Fig. 19) demonstrated that the accumulation of hCG- α mRNA was enhanced in cells producing greatly elevated levels of

α -protein. The enhanced level of hCG- α transcripts may result from an increased rate of synthesis, a decreased rate of degradation, or a combination of these two processes. Since the dot blot hybridization reflects the level of transcripts at the time of cell harvest, it does not take into account earlier, possibly significant, transient fluxes. Such a transient flux of the mRNA has been reported for the induction of tyrosine aminotransferase by dibutyryl cAMP in rat livers (Noguchi et al., 1982). Thus, it is difficult to compare directly the dot blot data with the hCG- α protein data which measures the 3 day accumulation of subunit in the medium. However, these data do show that certain conditions mediate the induction of hCG- α , in part, at the messenger level. Table XI shows that combining butyrate with IdUrd enhanced α protein 18.9 fold and α mRNA about 8.8 fold. Similar increases of the messenger and corresponding protein levels were observed in cells grown with Btr + BrdUrd, Btr + Thd, Btr + theophylline, and Btr + HU. This is in agreement with Darnell (1984) who observed that butyrate enhanced the rate of transcription for hCG- α in HeLa cells. Thus, the data presented in Table XI and Fig 20, along with Darnell (1984), indicate one site of butyrate action is on the α messenger levels.

Another probable site of butyrate action is on the glycosylation of alpha subunit. The carboxylic acid enhances incorporation of [3 H] glucosamine and [3 H] fucose into the α subunits produced by Chang human liver cells (Morrow et al., 1983) and by HeLa cells (Cox, 1981a; McClure and Cox, 1984). Similarly, Firestone and Heath (1981) have suggested

that glycosylation is required for the induction of alkaline phosphatase by dibutyryl cAMP in mouse L cells. A dual effect on the same protein has been reported for dibutyryl cAMP which increased both the transcription and the translation of tyrosine aminotransferase in the rat liver (Noguchi et al., 1982).

The synergism between butyrate and the deoxynucleosides suggests that they are acting via different mechanisms (Table IV). While synergism was observed for the induction of hCG- α but not PAP in this study, Park (1981) observed synergism for the induction of both proteins. This difference may reflect differences in the maintenance of stock cultures (spinner vs. monolayer), variations in the preparation and assay of cell extracts, and/or the use of different medium (MEM vs. RPMI 1640). The strongest and most consistent synergism was between butyrate and theophylline (Fig 11). While theophylline alone occasionally induced hCG- α to an extent similar to butyrate alone, the combination of these two agents consistently had a much greater than additive effect. This synergism involved a decrease in the lag between the addition of the effector and the onset of induction (Fig 12). The presence of a lag is consistent with an effect on protein production per se and not merely on the secretion of an intracellular pool of preformed subunit. A decrease in this lag may be due to a potentiation of transcriptional regulation and/or to post-transcriptional processes such as secretion. The first possibility is implied by the enhanced level of hCG- α mRNA (Figs 19, 20, and Table XI). The possibility of a primary

effect on the synthesis of a regulatory protein or of an enzyme (e.g. glycosyltransferase) involved in the expression of hCG- α or PAP cannot be ruled out.

The effect of theophylline on hCG- α production is different from observations made by other groups. Hussa et al. (1977, 1978) found that dibutyryl cAMP and theophylline stimulated the eutopic production of hCG and subunits by normal term placenta and human malignant trophoblastic cells in vitro. The N⁶- and O^{2'}- monobutyryl derivatives of cAMP and the phosphodiesterase inhibitors, papaverine and 3-isobutyl-1-methylxanthine (IBMX), enhanced hCG secretion. However, these mediators of cAMP levels did not enhance the ectopic production of hCG or subunits in three non-trophoblastic cell lines: ElCo (established from a breast carcinoma and a producer of hCG- α), CaSki (established from a cervical carcinoma and a producer of hCG- β) and DoT (established from a spinal metastasis of a cervical carcinoma and a producer of hCG- β). Thus, HeLa cells differ from these other non-trophoblastic cell lines in that theophylline alone stimulated hCG- α production and was synergistic with butyrate (Table III).

Theophylline has been reported to inhibit constitutive PAP activity (Griffen et al., 1974) as well as that induced by IdUrd and hydrocortisone in HeLa cells (Wharton and Goz, 1979a). Wharton and Goz observed that caffeine and IBMX inhibited the induction of alkaline phosphatase with the order of potency being IBMX > theophylline > caffeine. These xanthine compounds decreased the heat stable form

relative to the heat labile form. The inhibition reported by Griffen was specific for theophylline and did not occur with either caffeine or xanthine. In addition, 1 mM theophylline inhibited partially purified PAP activity 50%, but the enzyme was not appreciably inhibited by caffeine or xanthine. In another study, theophylline at 0.1 mM inhibited partially purified calf intestinal alkaline phosphatase by 50% in an uncompetitive manner as well as human alkaline phosphatase activity in the sera of healthy individuals and patients with a variety of disorders (Fawaz and Tejirian, 1972). Fawaz and Tejirian did not observe an inhibition of alkaline phosphatase by caffeine or theobromine. Table III shows that xanthine compounds, in general, inhibited PAP activity in HeLa cells with a greater effect on induced than control activity. The relative potency was theophylline > caffeine > theobromine > xanthine. These results are consistent with the work of Wharton and Goz (1979a) but not with Griffen et al. (1974) or Fawaz and Tejirian (1972). The inconsistencies may be due to differences in species, isozyme, or HeLa cell strain. Although, the work of Fawaz and Tejirian indicates that theophylline can interact directly with alkaline phosphatase to inhibit activity, other mechanisms may be involved. For example, theophylline, as well as caffeine and adenine, inhibited the cAMP induction of tyrosine aminotransferase (TAT) in the HTC rat hepatoma cell line by enhancing the rate of enzyme degradation (Stellwagen, 1974).

Although theophylline and other methylxanthines inhibit cyclic nucleotide phosphodiesterase, cAMP levels do not appear to be significant in the production of hCG- α and PAP activity. Figure 11 shows that 1 mM cAMP alone did not induce hCG- α and in combination with 1 mM theophylline induced hCG- α about 4 fold. The magnitude of this stimulation does not appear to be sufficient to account for the synergism observed when theophylline is combined with butyrate. Whereas theophylline, caffeine, and theobromine inhibit phosphodiesterase, only theophylline enhanced the butyrate induction of hCG- α (Table III). Furthermore, butyrate had little effect on the level of cAMP (Table X). Theophylline increased cAMP by about 1.7 fold and butyrate and theophylline about 1.8 fold. The lack of a significant change in cAMP may be due to measuring levels after prolonged exposure (72 hours) to these compounds. A significant, transient increase may have been observed if cAMP levels were monitored continuously after the addition of the effector(s). However, Hilz et al. (1975) observed that after 24 hours, 3 mM theophylline increased cAMP levels only about 1.6 fold even though phosphodiesterases in vitro as well as in intact cells were effectively inhibited. These authors suggest that most of the cAMP in HeLa cells is strongly bound to specific proteins and thereby protected from phosphodiesterases. In addition, Wharton and Goz (1979b) reported that treatment of HeLa cells with 1 mM caffeine and theophylline for 72 hours resulted in a slightly elevated and constant concentration of cAMP after the initial hour of exposure. While Wharton and Goz observed a

correlation between the inhibition of PAP activity and the concentration of cAMP, the inducers IdUrd and hydrocortisone did not alter the concentration of cAMP. Also, Hart et al. (1980) concluded that the parathyroid hormone (PTH) inhibition of the induction of alkaline phosphatase by IdUrd in HeLa cells was not mediated by cAMP. PTH did not alter adenylate cyclase activity even though PTH activates adenylate cyclase in bone and kidney. In addition, parathyroid extract did not potentiate the increase in cAMP by IBMX. The PTH data was interpreted to mean that either cAMP does not inhibit PAP activity or the mechanism of PAP inhibition differs between PTH and alkylxanthines. Since phosphodiesterases also hydrolyze cGMP, the concentration of this cyclic nucleotide may be important.

Although the elevation in cAMP by alkylxanthines has been commonly attributed to its inhibition of phosphodiesterases, recent evidence suggests that the actual target may be the extracellular adenosine receptors to block the action of adenosine on adenylate cyclase (Daly et al., 1981, Fredholm, 1980).

Another action of methylxanthines is on cellular Ca^{2+} homeostasis (Kopf et al., 1984 and references therein). In this regard, it is of interest that the influx of Ca^{2+} in HeLa cells is stimulated by PTH (Borle, 1968) and IBMX (Friedman et al., 1976), compounds which also inhibit alkaline phosphatase induction in HeLa cells (Hart et al., 1980). It was also reported by Hart et al. (1980) that the calcium ionophore A23187 at about 1 μM significantly inhibited the IdUrd induction of alkaline phosphatase.

Although the mechanism by which xanthine compounds lower PAP activity is not understood, possible factors include cAMP, adenosine, Ca^{2+} , and/or a direct interaction with the enzyme. A concentration study may differentiate between effects on phosphodiesterase and adenosine receptors since the latter is effected by micromolar concentrations while the former requires millimolar levels (Daly et al., 1981, Londos et al., 1981). It would also be of interest to examine the effect of IBMX on butyrate induction. While Wharton and Goz (1979b) observed a correlation between cAMP levels and the inhibition of alkaline phosphatase activity, a mechanism connecting these two events has not been delineated. Also, the lack of effect on cAMP levels by PTH, IdUrd, and hydrocortisone argue against a cAMP mediated regulation of alkaline phosphatase activity. The role of Ca^{2+} or adenosine in PAP activity has not been studied.

Deoxycytidine inhibited the induction of hCG- α and PAP by a number of compounds (Table IV), although the the data presented in Fig 15 suggest that the butyrate induction of hCG- α may be less sensitive. These data extend the observations of Goz et al. (1980) that dCyd inhibits the induction of PAP by IdUrd, hydrocortisone, butyrate, and choline chloride and are the first report of its inhibition of α subunit induction. In addition, Davidson and Kaufman (1977) reported that dCyd reversed the suppression of pigmentation in Syrian hamster melanoma cells by BrdUrd. Both Goz et al. (1980) and Davidson and Kaufman (1977) suggest that dCyd does not alter gene expression by interfering with

overall incorporation of IdUrd or BrdUrd into the DNA, although incorporation into specific regions may be relevant.

The effect of dCyd on the induction of hCG- α and PAP by butyrate suggests that dCyd interferes with an early event of butyrate induction. Once butyrate initiates this process, it cannot be reversed by dCyd (Fig 17). The greater sensitivity of PAP induction to dCyd is consistent with the concept that PAP and hCG- α are not co-ordinately regulated. Furthermore, it suggests that dCyd interferes with a process more closely associated with the induction of PAP than the induction of hCG- α . While dCyd had a preferential inhibition of PAP induction, dGuo and possibly dAdo inhibited the butyrate induction of both PAP and hCG- α (Table VIII). The relative potencies of the deoxynucleosides to inhibit induction was dGuo > dAdo > dCyd. Thymidine was unique in that it either had no effect on butyrate induction (PAP) or was synergistic with butyrate (hCG- α).

Taken together, these data suggest that nucleosides or nucleotide compounds may be involved in regulating the expression of hCG- α and PAP. Although the mechanism is unknown, there are several possible sites of action. The incorporation of deoxynucleoside analogs (IdUrd or BrdUrd) into the DNA may alter the binding of proteins which regulate gene transcription. BrdUrd-substituted DNA has been demonstrated to result in a tighter binding of the lac repressor protein (Lin and Riggs, 1971, 1972) and other proteins (Gordon et al., 1976). Bick (1977) reported that dCyd expanded the intracellular pool of TTP which decreased the

incorporation of BrdUrd into DNA, thereby reversing the inhibition by BrdUrd of the butyrate-mediated differentiation of Friend murine leukemia cells.

However, other mechanisms are also possible. For example, BrdUrd induces prolactin synthesis in GH cells via gene amplification (Biswas and Hanes, 1982) of an extra chromosomal element (Wilson et al., 1983). Also, the inhibition of pigmentation in melanoma cells by BrdUrd appears to involve a mechanism independent of its incorporation into DNA (Davidson and Kaufman, 1977). Furthermore, dCyd inhibits the induction of PAP by hydrocortisone, butyrate, and IdUrd in HeLa cells (Goz et al., 1980). While dCyd may be affecting the incorporation of IdUrd into DNA, this explanation does not account for its inhibition of induction by butyrate and hydrocortisone. Recently, hydroxyurea, a long-established inhibitor of ribonucleotide reductase, has been shown to inhibit the degradation of internalized epidermal growth factor (Masuda et al., 1982). The data of Chou and Robinson (1977a), Davidson and Kaufman (1977), and Goz et al. (1980) indicate that the free forms of the deoxynucleosides alter gene expression, suggesting that deoxynucleotide pool sizes may be important.

Deoxynucleotide pool imbalances are known to be mutagenic in mammalian cells presumably by decreasing the fidelity of DNA replication (Meuth et al., 1979; Weinberg et al., 1981; Kunkel et al., 1982; Peterson et al., 1983). In the present study, butyrate decreased the pool sizes of dATP and TTP. Also, Dexter et al. (1981) observed that

butyrate, as well as N,N-dimethylformamide, modulated the activities of purine metabolizing enzymes in cultured human colon carcinoma cells in a cell-type dependent manner. For example, butyrate increased the activity of adenosine deaminase about 5 fold in HCT-15 cells but decreased the activity of the enzyme about 2 fold in cells of DLD-1 clone A. Depletion of a key nucleotide may inhibit DNA synthesis and synchronize the cell population at the G1/S border. Many of the inducers have been characterized to synchronize cells near the G1/S border. If active genes are preferentially replicated early in S (Kajiura and Mueller, 1964, Furst et al., 1981), such a cell cycle block may enhance the accessibility of the gene to RNA polymerase. Evidence for a relationship between the time of replication and gene activity has been reviewed by Taylor (1984). However, Darnell (1984) has demonstrated that butyrate induces the transcription of hCG- α independent of the position of the cell in the cell cycle. Also, the inability of butyrate to induce the autopic production of hCG- α appears inconsistent with a cell cycle related mechanism. All of the deoxynucleosides inhibited DNA synthesis and the combination of effectors inhibited DNA synthesis to a greater extent than an individual effector by itself. The apparent lack of correlation between the degree of DNA synthesis inhibition and the strength of induction seem inconsistent with the idea that these two events are dependent on each other.

While these various reports relate deoxynucleosides to DNA synthesis, deoxynucleoside analogs may alter processes involving nucleotides which are unrelated to DNA replication as illustrated by the recent identification of a cytosine arabinoside nucleotide sugar compound in cells grown in 3 μM cytosine arabinoside (Lauzon et al., 1978a, Lauzon et al., 1978b). Thus, one possibility would be an effect of deoxynucleosides on nucleotide sugars which are involved in membrane and glycoprotein biosynthesis. It is interesting that butyrate inhibited the incorporation of exogenous [^{14}C] choline (Fig 18). This inhibition may result from a dilution of the label due to enhanced phosphatidyl choline synthesis (via CDP-choline) or degradation of choline containing compounds, an inhibition of the uptake of exogenous choline, or a demethylation of choline which would remove the radiolabel.

Several pieces of information make an effect on nucleotide sugars attractive: 1) induction is inhibited by deoxynucleosides at 2 mM but not at 100 μM , while micromolar concentrations of exogenous deoxynucleosides significantly alter deoxynucleoside triphosphate levels in human T lymphocytes (Cohen et al., 1983), 2) lack of a stringent correlation between induction and the inhibition of DNA synthesis, 3) the role of nucleotide sugars in the glycosylation of glycoproteins and the biosynthesis of membrane components, 4) methotrexate and FdUrd appear to expand the dUTP pools by inhibiting thymidylate synthetase resulting in the formation of dUDP-GlcNAc in lymphoid cells, and a

compound synthesized by cells treated with FdUrd has been tentatively identified as 5-FdUDP-GlcNAc (Peterson et al., 1983), 5) the modulation of alkaline phosphatase activity by choline, lysophosphatidyl choline, and Rosenthal's inhibitor, suggesting that the membrane composition may be important in regulating alkaline phosphatase activity (Hung and Melnykovych, 1976; Melnykovych and Lopez, 1977), 6) the many membrane associated changes induced by butyrate, and 7) the previously mentioned significance of glycosylation to the induction of hCG- α by butyrate (Morrow et al., 1983; Cox, 1981a; McClure and Cox, 1984) and of PAP by dibutyryl cAMP (Firestone and Heath, 1981).

A correlation between hypomethylation of DNA and gene activity, in general, has been suggested by others and has been reviewed by Doerfler (1983) and Bird (1984). Recently, methylation of the hCG- α gene was examined in DNA isolated from HeLa 2.1 (a low α producer), HeLa 2.2 (a high producer), and human placenta using the restriction endonucleases Hpa II and Msp I and a radiolabelled α -cDNA probe. Both enzymes recognize the four base sequence of 5'-CCGG-3', but Hpa II does not cleave the DNA when the internal cytosine is methylated whereas Msp I will cut regardless of methylation. This experiment revealed that the α gene is methylated to a greater extent in 2.1 DNA than in 2.2 DNA and further suggested that the gene structure of α in HeLa DNA differed from the normal placental gene (Clare Kenney and G. Stanley Cox, Dept. of Biochem., U. of Neb. Med. Center, Omaha, NE, personal communication). Thus, the difference in the rate of α production among the HeLa cell lines may be explained, at least in part, by the degree of methylation of the alpha gene.

The data presented in this study may be explained by effects of butyrate and the deoxynucleoside inducers on transcription and glycosylation but via different mechanisms. For example, the modification of chromatin proteins (acetylation, phosphorylation, ADP-ribosylation) mediated by butyrate (Boffa et al., 1981) probably affects their binding to DNA and alters the chromatin structure resulting in enhanced gene activity. However, deoxynucleoside inducers may act by changing the base composition of the DNA, either by incorporation of the modified nucleoside itself or by promoting misincorporation, which could alter protein binding and chromatin structure leading to gene activation. At the level of glycosylation, butyrate could alter the level of enzyme or modify the activity of glycosyltransferases such as sialyltransferase I (Fishman et al., 1974) or galactosyltransferase (C. Stanley Cox, unpublished observations). Deoxynucleosides may also change the activity of glycosylation enzymes, perhaps as a result of either presenting the enzyme with a modified substrate (deoxynucleotide instead of ribonucleotide sugar) or possibly acting directly as a positive or negative modulator.

This mechanism could explain the time course of induction data in Fig 14 since changes in the chromatin structure resulting from protein modification would presumably occur faster than those resulting from alteration in the base composition. Since different mechanisms are involved, the combination of butyrate with a deoxynucleoside inducer may be synergistic. Also, deoxycytidine appeared to be a stronger

antagonist of the deoxynucleoside inducers than of butyrate (Table IV). In this case, deoxycytidine may alter pool sizes to preferentially counter the deoxynucleoside effects.

A number of experiments could be designed to test this hypothesis.

- 1) As an initial experiment it would be beneficial to use specifically labelled compounds to determine how the deoxynucleosides are metabolized (phosphorylation, dehalogenation, base replacement by salvage pathway enzymes).
- 2) Look for incorporation of the deoxynucleoside inducer into the DNA using radiolabelled precursor incorporation into TCA precipitable material of purified DNA and/or determination of the DNA base composition.
- 3) Look at the incorporation into DNA as a function of inducer concentration and time course on induction.
- 4) Examine the incorporation of radiolabelled precursors into the α gene region by hybridization to α -cDNA.
- 5) If the chromatin structure is altered to increase the accessibility of the α gene to transcription proteins, this may be detected by following the kinetics of nuclease digestion of this region.
- 6) Monitor the activity of glycosylation enzymes with the time course of induction.
- 7) Examine the rate of glycosylation of α and PAP in the presence of various inducers.
- 8) Study the half-life and rate of secretion or transport of α and PAP in relation to induction.
- 9) If the incorporation of the halogenated deoxyuridine compounds into DNA is required for induction, then dCyd would not be expected to block induction in those cells preincubated with the inducer. A partial or complete inhibition may be expected if the site of action is

glycosylation where dCyd would still presumably generate a competing compound.

The data in this study also suggest that the expression of hCG- α and PAP in HeLa cells are not co-ordinately regulated. This conclusion is supported by the lack of correlation between the two genes in numerous HeLa cell lines with regard to constitutive and butyrate induced synthesis (Table I, Fig 1), the difference in inducer concentration which stimulated their maximum production (Fig 10 and 13), the synergism observed between butyrate and other inducers for the induction of hCG- α but not PAP (Table IV), the inhibition of PAP induction but not hCG- α induction by xanthine compounds (Table III), and the observation that the butyrate induction of PAP activity is more sensitive to dCyd than the induction of hCG- α (Fig 15).

Since the study of PAP induction involved the measurement of activity, it is not known if the observed effects are on the synthesis of protein or the catalytic efficiency of the enzyme. The delay in induction (Fig 14) along with the reports of Hamilton and Sussman (1981) and Ito and Chou (1984) argue for increased synthesis. If regulation of gene expression is restricted to the transcriptional level, the discordant expression of these proteins implies that there may be a difference in the chromatin organization or promoter strength of these genes (Weisbrod, 1982). Perhaps a difference in chromatin proteins associated with each gene or distribution of specific bases within each gene or near control sequences could result in different sensitivities

to the same inducer. If a post-transcriptional effect is being observed, the discordant regulation may then be related to the difference in the fate of each protein, i.e., secretion for α or insertion into the plasma membrane for PAP. Although most evidence indicates that secretion and transport of plasma membrane proteins to the surface occur via the same pathway, Gumbiner and Kelly (1982) suggest that in some instances these two processes may diverge. It is also possible that the primary action of the inducers is on a membrane component which alters PAP activity (such as phosphatidylcholine). Membrane composition has been shown to be important to the activity of other membrane enzymes (Merisko et al., 1981; Galo et al., 1981; Hegyvary et al., 1980).

As part of a continuous project, this study indicates that deoxynucleosides may be useful in determining processes important to hCG- α production and PAP activity in HeLa cells and perhaps to gene expression in general. In this regard, mutants of deoxynucleoside metabolism may help determine which metabolites are significant. Also, the possibility of multiple effects by some of these agents does not seem unreasonable. Further experiments are needed to better understand the action of these compounds on gene expression.

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